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ARSENIC IN THE SUSPENDED MATTER OF TOWN AIR.

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METHOD.

THE suspended matter is collected by drawing a known volume of air through a circular area on a sheet of filter-paper which is replaced by a fresh one after 24 hours (for further details see Waller, 1952). The discoloured area was cut out and the specimens thus obtained during one month were combusted together in one or two batches with H_2SO_4 (5 to 15 ml.) and HNO_3 in successive amounts (usually 30 to 40 ml. in all); when the solution was colourless two successive portions of 25 ml. water were added and the mixture boiled until white fumes appeared, to remove nitric acid. The analysis was completed by the Gutzeit method. A blank estimation was carried out on the reagents and in the latter half of the investigation a weighed amount of the filter-paper surrounding the discoloured areas was included in the blank, but this addition makes no considerable difference. Occasional very high blanks were discarded as due to some unusual contamination. The amounts stated below and in Table I and Fig. 1, 2 and 3 represent the total amount of arsenic, as μg. As_2O_3, found in the smoke papers for one month, calculated per cubic metre of the air drawn through those papers.

No high degree of accuracy is claimed for these results as the amount of arsenic found may be only twice that present in the blank, which latter amount ranges usually from *nil* to 2μg. The error of these estimations cannot easily be lessened by taking larger quantities, as the combustion of such amounts of paper becomes difficult. But the method probably gives for the present purpose a sufficient measure of a very variable quantity.

RESULTS.

(1) The monthly means (Table I) from the eight localities all together show (Fig. 1) amounts in November, December and January (mean 0·104) which are about twice as great as those found during the rest of the year (mean 0·055). This seasonal change is less than that, namely about 3·5 times, found in the case of benzpyrene (Waller, 1952), which difference suggests that arsenic is less predominantly a contribution from domestic smoke.

TABLE I.—*Arsenic in Suspended Matter.*

Each figure in columns 1 to 12 represents the total amount of arsenic, as μg. As_2O_3, found in the smoke papers for one month, calculated per cubic metre of the air drawn through those papers. The localities are arranged in descending order of the means in column 13.

Town.	1 Jan.	2 Feb.	3 March.	4 April.	5 May.	6 June.	7 July.	8 August.	9 Sept.	10 Oct.	11 Nov.	12 Dec.	13 Mean.
Beckton (London):													
1947	0·132	0·00	0·039	0·132	– 0·132	0·071	..	0·041	..	..	..	..	0·132
1948	..	..	..	..	..	..	0·115	(0·121 1948, 19 days)	0·330	..	0·339	..	
Bilston:													
1946	..	..	..	..	..	..	..	..	..	..	..	0·112	
1947	0·099	0·036	0·036	..	..	..	..	..	..	..	..	..	0·075
1948	0·108	..	..	..	..	..	..	..	..	..	..	..	
1949	..	..	..	0·024	0·064	0·086	0·059	0·041	0·075	..	0·050	..	
Manchester:													
1948	0·019	..	..	0·092	0·028	..	0·087	0·057	0·119	0·041	..	0·137	0·073
Liverpool:													
1948	0·132	0·016	..	0·094	0·091	0·048	0·07	0·074	0·021	0·035	0·101	..	0·068
Sheffield:													
1947	0·070	..	..	0·080	0·030	..	..	..	0·034	0·150	0·060	..	0·065
1948	0·094	0·096	..	..	..	0·073	0·020	0·019	..	..	..	0·115	
County Hall (London):													
1947	0·096	0·031	..	0·00	..	0·020	0·00	0·127	0·048	0·00	0·074	0·127	0·058
1948	0·077	..	..	..	0·092	..	..	..	..	..	..	..	
Hull:													
1948	..	..	..	0·011	0·057	0·021	0·047	0·053	0·051	0·070	0·110	0·064	0·054
1949	0·114	0·014	0·039	..	..	..	..	..	..	..	..	..	
Bristol:													
1948	..	0·079	..	..	0·019	0·036	0·049	0·034	0·018	..	..	..	0·037
1949	..	..	..	..	..	..	..	..	0·012	0·025	0·047	0·049	
Mean	0·094	0·039	0·038	0·049	0·064	0·051	0·056	0·063	0·079	0·053	0·117	0·101	

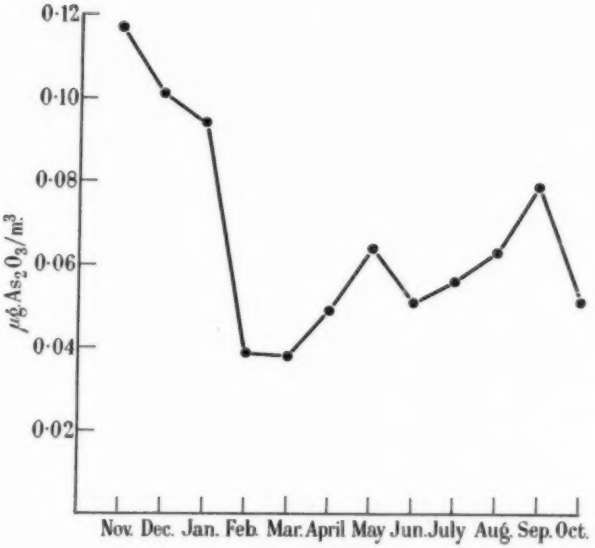

FIG. 1.—Mean seasonal variations in arsenic content of air at eight stations.

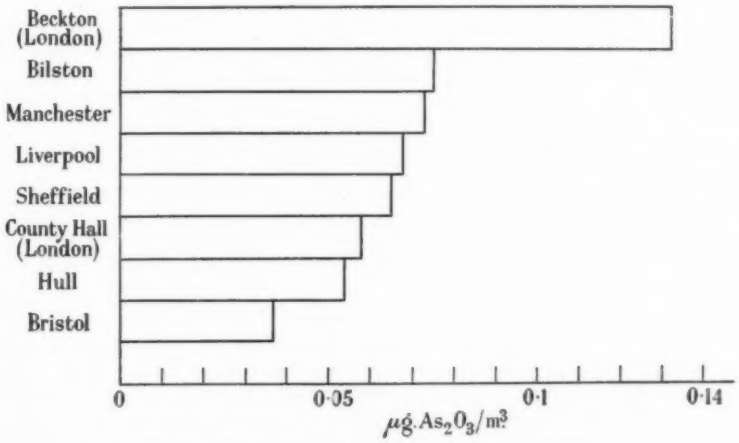

FIG. 2.—Mean arsenic content of air at eight stations.

(2) The results from the various stations (Fig. 2) show a maximum (Beckton 0·132) which is 3·5 times as great as the minimum (Bristol 0·037). Sheffield, Manchester, Liverpool and Bilston form a group showing a mean amount (0·07) which is about one-half that found at Beckton and about 25 per cent more than is shown by Hull (0·054) and by London as represented by County Hall near the centre of the city (0·058) and is twice as great as that at Bristol (0·037). No claim is made that the data for winter and summer (Fig. 3) are sufficiently numerous to

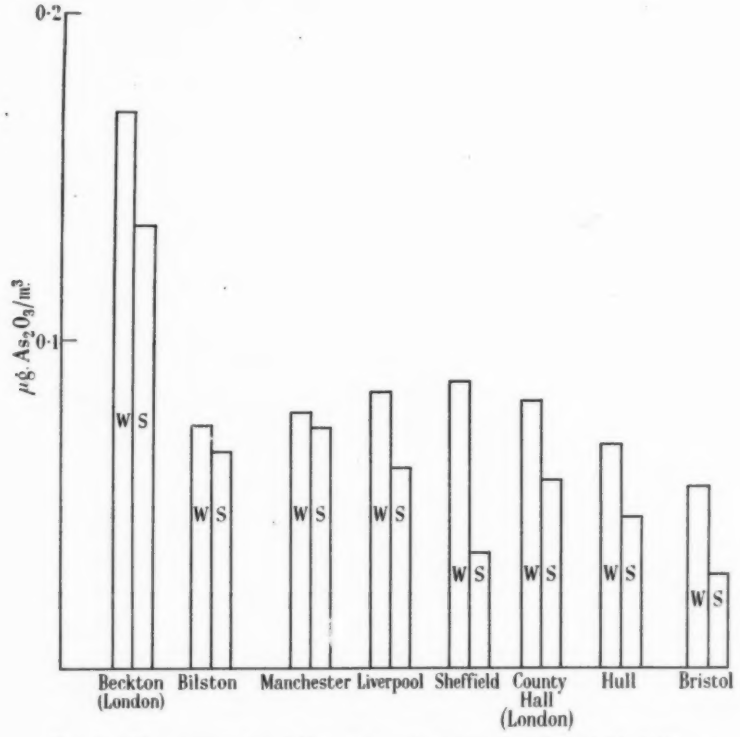

FIG. 3.—Mean seasonal variations in arsenic content of air at eight stations.
W = winter (November to March); S = summer (May to September).

establish any exact comparison between the seasonal changes in the individual towns. Dr. Richard Doll has pointed out to us that the average values for the different stations would be more properly comparable, in view of the seasonal variations, if (1) the means of two figures for the same month and town were taken to represent that month, and (2) the mean of the figures for two months which are separated by a gap of one month without data were taken to represent that month. When this is done the figures in Column 13 of Table I become—Beckton 0·162, Liverpool 0·072, Sheffield 0·071, Manchester 0·070, Bilston 0·063,

Hill 0·054, County Hall 0·053, Bristol 0·043. The result of the change, is to accentuate the difference between Beckton and the other stations.

The inherent difficulty in any attempted correlation of atmospheric pollution with the prevalence of any disease is that the former is, in practice, measured only at a very few stations in the whole area which the population in question occupies, and at these points one may or may not secure fair samples.

The volume of air breathed by the " standard man " in 24 hours is estimated to be about 20 cubic metres. If this air contained 0·07 μg./m.^{3} As_2O_3 he will inhale 1·4 μg. in 24 hours, or 0·5 mg. in a year, or 35 mg. in 70 years. A person who smokes cigarettes each of which contains 50μg. As_2O_3, of which 10 per cent escapes in the smoke (Daff and Kennaway, 1950), would volatilise this yearly amount (0·5 mg.) in smoking 100 cigarettes ; the maximum official dose of Fowler's solution (0·5 c.c.) contains 10 times this amount (5 mg.).

The association of smoking with cancer of the lung, together with the higher incidence of this form of cancer in towns (Stocks, 1936, 1949 ; Kennaway and Kennaway, 1951) might perhaps be due not only to different smoking habits in town-dwellers, but also to a summation of carcinogenic factors, of which something connected with smoking is by far the most potent, while agents in town dust, such as benzpyrene and arsenic, might have a supplementary effect.

We have little, if any, data about such summation in man, of carcinogenic actions apart from the instance of xeroderma pigmentosum. The summation of the effects of two carcinogens (" syncarcinogenese " of Bauer, 1949) would not be easy to distinguish, in the case of weak agents, from co-carcinogenesis. One might suggest some such classification as the following of the not very abundant data available upon the types of summation (Table II) ; no claim is made that this is in any way a complete summary of the literature. The summation of action of two chemical carcinogens is not easy to demonstrate, and we appear to have few indubitable instances of it.* In connection with the subject of cancer of the lung, the work of Lynch (1935) and Andervont (1937) upon summation in the lung of some strains of mice is of especial interest, although the neoplasms of the lung in this species differ from those in man. The evidence for the carcinogenic action of arsenic in the human lung was summarised in an earlier paper (Kennaway and Kennaway, 1947) ; see also Hill and Faning (1948).

SUMMARY.

The arsenic content of the air in eight towns in England has been estimated at various seasons of the year. A range in these quantities of about three-and-a-half fold was found and the concentration in winter was about twice that in summer. These amounts are compared with those which might be liberated in cigarette smoking, and a possible summation of carcinogenic effects is discussed.

We wish to thank the Medical Research Council, the British Empire Cancer Campaign and the Anna Fuller Fund for grants which have enabled us to carry out this investigation. Our indebtedness to the local authorities who have supplied the material for this investigation is recorded in the following paper (Waller, 1952).

* Since this paper was written the synergistic action of pairs of compounds upon the liver and ear-duct of rats has been described (MacDonald, Miller, Miller and Rusch, 1952).

TABLE II.—*Types of Summation of Carcinogenic Action.*

Author.	Carcinogenic factors.				Tissue.	Species.
	Chemical.	Radiation.	Virus.	Genetic.		
..	..	Sunlight	..	+	Epidermis, xeroderma pigmentosum	Man.
Mayneord and Parsons (1937)	Na-1:2:5:6-dibenzanthracene-9:10-endo-$\alpha\beta$ succinate	X-rays	..	..	Subcutaneous	Mouse.
Lynch (1935)	1:2:5:6-dibenzanthracene	..	..	+	Lung	Bagg albino mouse.
Strong, Smith and Gardner (1938)	3:4:5:6-dibenzcarbazole	..	..	+	Liver	Mouse, CBA male.
Andervont (1937)	1:2:5:6-dibenzanthracene	..	..	+	Lung	Mouse, Strain A
Rogers and Rous (1951)	20-methylcholanthrene, or 9:10-dimethyl-1:2-benzanthracene	..	Shope	..	Epidermis	Rabbit.
Engelbreth-Holm (1941)	20-methylcholanthrene. ? natural oestrogen also	..	Bittner	+	Mammary gland	Mouse, dilute brown.
Gilmour (1937)	1:2:5:6-dibenzanthracene and oestrone	..	..	..	Epidermis	Mouse.

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THE BENZPYRENE CONTENT OF TOWN AIR.

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THE use of the fluorescence spectrum by Mayneord and Hieger led to the detection of 3-4-benzpyrene in coal tar and to the isolation of this compound from pitch (Cook, Hewett and Hieger, 1933). Recently Goulden and Tipler (1949) identified it by the same means in domestic soot, which is carcinogenic (Passey, 1922), and their method has been extended to samples of smoke drawn from the atmosphere. By collecting the daily samples taken by local authorities in a number of different areas during several years, it has been possible not only to identify benzpyrene in all samples examined, but to observe its variation with time of year in any one place, to gain an idea of the variation between different places, and to deduce the nature of the principal source of it.

COLLECTION OF SAMPLES.

Under the guidance of the Department of Scientific and Industrial Research an increasing number of local authorities in large and small towns throughout the country has, during the past few years, collected daily samples of suspended impurity at fairly central points in their respective areas as records of the trends in smoke concentration from month to month and year to year. The apparatus is described fully in "Atmospheric Pollution in Leicester" (Leicester, 'Atmospheric Pollution,' 1945), and generally consists of a small electric motor drawing air from outside a building through a filter-paper, upon which most of the suspended smoke is deposited, followed by a bottle containing H_2O_2 for the determination of SO_2, and a gas meter to measure the volume of air. Filter-papers are changed daily, and the "shade" of the resulting stain, as compared with a calibrated "scale of shades", is taken as a measure of the smoke collected. These data are collated on monthly sheets showing the daily volume of air sampled, the shade number and smoke concentration deduced therefrom, with monthly summaries. By examination of each monthly set of filters, variations in the quality and quantity of smoke collected at that one station from month to month can be determined. A similar procedure at other stations may enable one, with all due reservations, to make some comparison between them. The smoke consists chiefly of carbon, with some other inorganic matter, and a variable amount of tarry matter, containing a complex mixture of hydrocarbons. In this series of experiments attention was focused on benzpyrene, and no serious attempt was made to identify any other hydrocarbons.

Over 60 stations in Great Britain make observations of atmospheric pollution by this method; filters from the following ten were used in this investigation.

The figures in brackets after the name of the town give the population in round numbers:

Bilston (33,000): on the first floor, in a residential and industrial district. The town lies in a heavily industrialised area with several larger towns nearby.

Bristol (442,000): on the second floor, in a busy main thoroughfare, and close to a branch-line railway station and coal yard. Pollution from other towns is unlikely.

Burnley (85,000): in the old gas-works yard in the town centre, surrounded by mill chimneys. There are large industrial areas to the south, south-west and east of it.

Cannock (41,000): near the centre in a commercial and residential district, with coal mines around the town, which lies just north of a large industrial area. Distance from Bilston 9 miles.

Hull (300,000): at about 14 ft. above a main road near the centre of the city, which is well to the east of any other large industrial area.

Leicester (285,000): at 40 ft. above a busy road near the centre in a district consisting mainly of offices. The city is fairly well isolated from other industrial areas.

London (Beckton): on the first floor at the London County Council Northern Outfall Works just to the south-east of Beckton Gas Works, but fairly isolated from all other buildings, 9 miles east of County Hall.

London (County Hall): at 75 ft. above ground level in a commercial district, and roughly at the centre of the Greater London area (population 8,346,000).

London (Crossness): near ground level in an open position at the London County Council Southern Outfall Works, with very few adjacent buildings, 11 miles east of County Hall and 2 miles east-south-east of Beckton.

Sheffield (513,000): in a manufacturing district in the centre of the city, with factory chimneys nearby.

PREPARATION OF MATERIALS.

The risk of contamination in the detection of carcinogens has been emphasized by several workers (Earle, 1943; Hieger, 1946; Anderson, 1947). In the present series of estimations of very small quantities of benzpyrene by fluorescence methods the utmost care had to be taken throughout. A high degree of "fluorescence purity" was required of solvents which had to be evaporated down to small volumes. These were redistilled until not only the distillate, but also the residue, showed no fluorescence in ultra-violet light. The fluorescence spectrum of benzpyrene was identified in most first residues from both benzene and petroleum ether. All glass apparatus, together with Soxhlet thimbles and any other equipment, was refluxed for several hours before use with acetone to remove any trace of oil, grease or dust.

METHOD OF ESTIMATION.

The smoke filters were cut up, packed into thimbles, and extracted in a Soxhlet apparatus with a suitable solvent. In preliminary experiments a mixture of equal parts of benzene and petroleum ether was found to be very satisfactory, as it seemed to extract most of the benzpyrene whilst leaving behind some unwanted substances. However, some of the benzpyrene may be adsorbed upon solid particles in the smoke, and in some cases a second extraction with a more polar solvent

extracted up to 50 per cent more benzpyrene. In the main experiment acetone was used, and each extraction was continued until (after about 4 hours) no further fluorescent material could be extracted. The resultant golden-yellow solution was passed through a short column of activated alumina (3 × 1·4 cm., Spence Type H), which removed any finely divided particles, traces of water, and certain of the unwanted hydrocarbons. After running more acetone through the column the resultant solution was evaporated, leaving a yellow oily droplet with a brilliant blue-violet fluorescence in ultra-violet light, which was dissolved as far as possible in several successive lots of boiling petroleum ether (60–80°); the insoluble part was redissolved in acetone and retained separately to be checked for the absence of benzpyrene. The petroleum ether extracts were pooled, evaporated almost to dryness and made up to 20–40 ml. in fresh petroleum ether. This ensured the removal of any trace of acetone left over in the original residue, which was essential for the success of the chromatogram which was to follow. The solution at this stage was pale straw-coloured, with a strong bluish fluorescence in ultra-violet light, and on passing through a column of alumina (8 × 1·4 cm.) previously washed with petroleum ether, most of the fluorescent material was adsorbed as a narrow, blue-fluorescing band near the top of the column.

Development with petroleum ether, followed by a mixture of 30 per cent benzene in petroleum ether, produced a series of rather diffuse bands, distinguishable in ultra-violet light by their slightly differing fluorescence. A pale blue band moved down the column fairly rapidly with the 30 per cent mixture, and was collected as eluate 30% A. This was followed by a blue-violet band collected as 30% B, and a further blue band collected by elution with 50 per cent mixture. After carrying out this procedure on a whole series of smoke filters for various periods and places, the fluorescence spectra of each set of 3 eluates were photographed along with that of a standard solution of pure benzpyrene (Fig. 1).

Solutions were contained in a small glass cell illuminated at 90° by ultra-violet light from a General Electric Co. " Osira " lamp with separate Wood's glass filter, and spectra were recorded on a Hilger E.517 spectrograph, using a 0·1 mm. slit and 2-minute exposures with Ilford " Zenith " plates. All final solutions were prepared in petroleum ether, and no precautions were taken against oxygen quenching (Bowen and Williams, 1939; Miller and Baumann, 1943; Weil-Malherbe, 1944).

Schoental and Scott (1949) give the fluorescence bands of benzpyrene in petroleum ether as a main system at λ 4032, 4272 and 4535, with subsidiary systems at λ 4082, 4310 and 4149, 4367. With the weak solutions used here, only those at λ 4032, 4082, and 4272 appeared in the negatives, and not all of these in reproductions. The group of Hg lines around λ 3650 appears in all photographs, and serves as a useful reference line. The position and intensity of the bands varies with the solvent (Chalmers, 1938; Sambursky and Wolfsohn, 1940), but at this stage the difference between petroleum ether and its mixtures with benzene is unimportant. In most cases, the spectrum of benzpyrene was visible in eluate 30% B and not in the others. These former solutions still contained much material other than benzpyrene, some of which fluoresces in the same wave-length region as benzpyrene. Where necessary the solutions were evaporated and rechromatographed, but the benzpyrene was not freed entirely from fluorescent impurities by this means. Finally, each benzpyrene-containing fraction was evaporated

and made up in petroleum ether to a known volume. All solutions were examined at a strength such that they fluoresced uniformly across the test cell (i.e., that they were too weak for appreciable re-absorption to occur). To estimate the amount of benzpyrene in each solution, a control experiment was made in which a known amount of benzpyrene was mixed with non-benzpyrene-containing impurities recovered from the main experiment and the whole procedure repeated. By preparing a series of successive dilutions from the final solution of this experiment, a set of standards was obtained with which the unknowns could be compared. The spectra of all solutions were then photographed together on one plate (Fig. 2) and the intensity of each unknown matched against one of the standards by visual comparison. The total amount of benzpyrene extracted from each set of smoke filters could thus be calculated, and hence its concentration in the air sampled, and proportion in the smoke collected, deduced. This method compensates for the slight loss of benzpyrene during the experiment, for a control on pure benzpyrene alone showed that the recovery was of the order of 80 per cent. A blank experiment, substituting the clean surrounds of the filter-papers for their black centres showed a trace of benzpyrene, but too little to estimate. Apart from extreme sensitivity to contaminants in working materials, the method proved consistent in a large number of estimations and controls carried out at different times, so that although the absolute values of the results cannot be guaranteed, comparisons between them should be valid.

RESULTS.

After trials to determine the most suitable methods and periods, the remaining smoke filters were dealt with in three groups, as follows :

(i) A monthly survey for 12 consecutive months at London (County Hall), Sheffield and Cannock, chosen to represent towns of greatly differing size (Table I).

TABLE I.—*Monthly Concentrations of Benzpyrene.*

	Concentration in air, μg./100m.³			Proportion in smoke, parts per million.		
	London (County Hall).	Sheffield.	Cannock.	London (County Hall).	Sheffield.	Cannock.
May, 1949	—	2·0	—	—	70	—
June, „	2·6		—	140		—
July, „	1·2	2·4	0·6	75	80	120
August, „	2·1	2·1		150	80	
September, „	1·4	3·3	1·1	80	100	150
October, „	4·4	5·8	2·7	150	130	290
November, „	8·0	5·6	3·1	160	130	290
December „	12·2	6·3	2·7	280	150	360
January, 1950	14·7	7·8	3·2	330	150	350
February, „	9·5	6·4	2·7	280	150	340
March, „	10·1	6·5	2·7	290	140	440
April, „	4·8	4·4	1·1	240	130	370
May, „	2·5	—	1·6	160	—	260
June, „	—	—	0·4	—	—	125

(ii) A survey over 3 consecutive years at London (Crossness), as a check on year-to-year variations (Table II).

TABLE II.—*Seasonal and Mean Annual Concentrations of Benzpyrene.*

Concentration in Air: μg./100m.³

		Bilston.	Bristol.	Burnley.	Cannock.	Hull.	Leicester.	London. Beckton.	London. County Hall.	London. Crossness.	Sheffield.
Seasonal values	Summer, 1948	—	—	1·3	—	—	—	—	—	—	—
	Winter, 1948–9	—	—	4·8	—	—	—	—	—	—	—
	Summer, 1949	—	—	1·4	0·9†	—	—	2·5	2·0	—	2·3
	Winter, 1949–50	—	—	3·5	2·9	2·5	—	12·2	10·4	—	6·5
	Summer, 1950	0·8	0·5	1·3	—	0·9	—	4·8	1·3	—	1·8
	Winter, 1950–51	3·5	2·1*	4·0	2·1	2·7	3·5	9·1	4·5	—	6·1
Ratio of winter to summer values	1948–49	—	—	3·7	—	—	—	—	—	—	—
	1949–50	—	—	2·5	3·2	—	—	4·9	5·2	—	2·8
	1950–51	4·4	4·2	3·1	—	3·0	—	1·9	3·5	—	3·4
	Mean	4·4	4·2	3·1	3·2	3·0	—	3·4	4·3	—	3·1
Mean annual values	May, 1947–Apr., 1948	—	—	—	—	—	—	—	—	1·7	—
	May, 1948–Apr., 1949	3·2‡	—	3·0	—	—	2·9§	—	—	1·6	—
	May, 1949–Apr., 1950	—	—	2·5	1·9	—	—	7·3	6·2	1·5	4·4
	May, 1950–Apr., 1951	2·2	1·3	2·7	—	1·8	—	7·0	2·9	—	4·0
	Mean	2·7	1·3	2·7	1·9	1·8	2·9	7·1	4·6	1·6	4·2

Proportion in Smoke: parts per million

Seasonal values	Summer, 1948	—	—	56	—	—	—	—	—	—	—
	Winter, 1948–9	—	—	116	—	—	—	—	—	—	—
	Summer, 1949	—	—	59	167†	—	—	185	123	—	81
	Winter, 1949–50	—	—	92	352	82	—	284	270	—	143
	Summer, 1950	54	146	71	—	50	—	268	108	—	83
	Winter, 1950–51	94	242*	141	140	69	139	234	123	—	120
Ratio of winter to summer values	1948–49	—	—	2·1	—	—	—	—	—	—	—
	1949–50	—	—	1·6	2·1	—	—	1·5	2·2	—	1·8
	1950–51	1·7	1·7	2·0	—	1·4	—	0·9	1·1	—	1·4
	Mean	1·7	1·7	1·9	2·1	1·4	—	1·2	1·7	—	1·6
Mean annual values	May, 1947–Apr., 1948	—	—	—	—	—	—	—	—	—	—
	May, 1948–Apr., 1949	105‡	—	86	—	—	205§	—	—	—	—
	May, 1949–Apr., 1950	—	—	76	260	—	—	235	196	—	112
	May, 1950–Apr., 1951	74	194	106	—	60	—	251	116	—	102
	Mean	90	194	89	260	60	205	243	156	—	107

* March, 1950, substituted for March, 1951.
† May and June, 1950, substituted for May and June, 1949.
‡ Twelve month period, April, 1948, to March, 1949.
§ Twelve month period, October, 1948, to September, 1949.

(iii) A seasonal survey over as many summers and winters as possible at all remaining stations; summer is defined as the five months May to September, and winter as November to March; the mean of the two periods is taken as approximately the mean annual value (Table II).

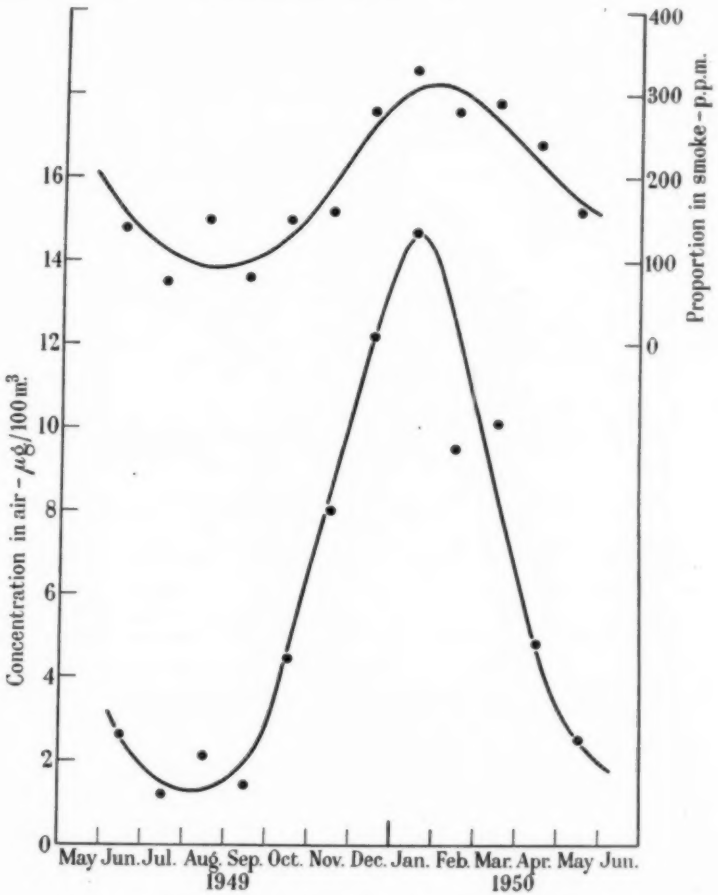

FIG. 3.—Yearly cycle of benzpyrene at London (County Hall).

The variations from month to month at any one station are greater than the difference between stations, so that a mean value over at least one year is required for any comparisons. Table II shows appreciable variations from one year to the next; an average over something like 5 years would be required to give reliable figures. As this is impracticable at present, mean annual values (Table II) representing averages over 1 to 3 years, must be taken as subject to a large margin of error.

† May and June, 1950, substituted for May and June, 1949.

§ Twelve month period, October, 1948, to September, 1949.

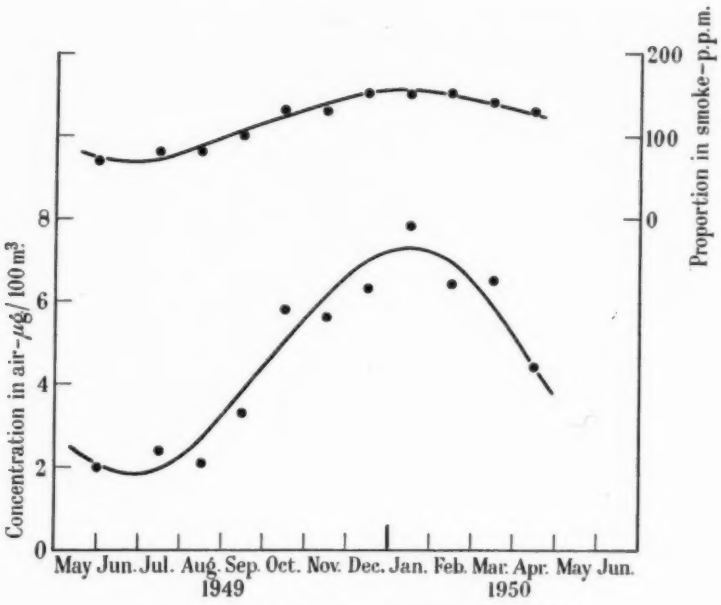

FIG. 4.—Yearly cycle of benzpyrene at Sheffield.

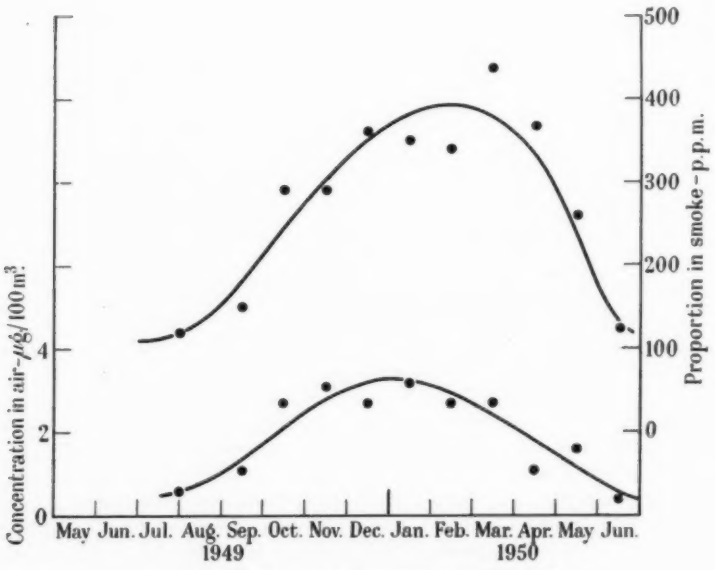

FIG. 5.—Yearly cycle of benzpyrene at Cannock.

A fairly well-defined yearly cycle is shown (Fig. 3, 4, 5), and although the positions of the maxima and minima vary slightly with the weather during the year in question, the cycle is repeated in much the same form year after year. A similar cycle is shown by the smoke as a whole (Leicester, 'Atmospheric Pollution,' 1945, p. 77), indicating the proportion of smoke from space-heating equipment, though the picture is complicated by the yearly cycle of weather conditions affecting the smoke concentration. The important point is, that not only does the concentration of benzpyrene show a distinct yearly cycle, but its proportion in the smoke does the same, indicating that smoke from some kind of space-heating equipment contains a higher proportion of benzpyrene than does the rest. Although power stations contribute to space-heating requirements, the proportionate rise in their smoke output in winter is small compared with that in the smoke from domestic fires. Gas works may also have a local effect (see below). Further, benzpyrene has been identified in domestic soot, but would not occur normally in the smoke from the more efficient use of coal in power stations and industrial undertakings, and not in any case from coke. Hence one of the chief contributors of benzpyrene to the atmosphere seems to be the domestic coal fire.

The range of yearly variation, defined here as the ratio of the maximum values shown by the curves in Fig. 3, 4 and 5 to the corresponding minimum values, is as follows:

	County Hall.	Sheffield.	Cannock.
Benzpyrene concentration in air	11·2	4·1	8·2
Benzpyrene as a proportion of smoke	3·5	2·2	3·5

County Hall, situated in the midst of the largest population, shows the greatest range of concentration. The summer minimum gives a rough upper limit to the general level due to industrial sources not subject to seasonal variation. Some years ago ('Atmospheric Pollution,' 1930, p. 7) the proportion of domestic to industrial smoke in London was estimated at 5 : 2, though it may well be lower now. A general discussion on recent changes in pollution is included in the 26th Report in this series ('Atmospheric Pollution,' 1949).

At Sheffield there is less variation during the year than at County Hall, suggesting the influence of industrial smoke containing a lower proportion of benzpyrene than domestic smoke, coupled perhaps with a greater use of domestic fires during the summer. The smoke at Cannock is primarily from coal mines and domestic fires and shows the highest proportion of benzpyrene, with a range intermediate between that of County Hall and Sheffield.

The seasonal values at the whole range of stations in the main confirm and extend the conclusions drawn above. Winter concentrations exceed summer ones by a factor of three or four times on the average, and with one exception the proportion of benzpyrene in the smoke is also greater everywhere in the winter by a factor of just under two. The highest values throughout are shown by Beckton, which should be regarded apart as a special case. Here there is much local pollution from the adjoining gas works, which would appear to contain at least as much benzpyrene as domestic smoke since the proportion of benzpyrene in the smoke persists at a level above the average throughout the year. However, the effects of such abnormal conditions fall off rapidly as the distance from the source increases, for at Crossness, only two miles away, the mean annual concentration is one of the lowest observed. These results serve also as a warning

that large variations in benzpyrene concentration can occur even within very short distances, so that to assess the mean concentration within a town would require readings from a large number of stations. Hence the position of a town in the following list, arranged in descending order of concentration, indicates only the conditions close to each station, and not necessarily those in the town as a whole:

	Mean annual concentration of benzpyrene in air—μg./100m.^{3}
London (County Hall)	4·6
Sheffield	4·2
Leicester	2·9
Burnley	2·7
Bilston	2·7
Cannock	1·9
Hull	1·8
Bristol	1·3

Beckton and Crossness have been omitted as not being centrally situated. One might expect the concentration near the centre of a town to bear some relation to the population, although many other local conditions play their part. In fact, the first six towns are very nearly in descending order of population, and the low position of the large cities of Hull and Bristol is perhaps noteworthy. Results from stations in many other towns not investigated might well be higher than most of those quoted. A short series of filters from central Manchester has shown a very high concentration, but it has not been possible to examine a whole year's filters from this station.

During a fog the concentration of benzpyrene, along with all other pollutants, reaches a high value, so that its proportion in the smoke is not greatly affected. To test this, all filters from days when the smoke concentration exceeded 1·25 mg./m.^{3} (called "Z" days, and in this case decidedly foggy) were selected from one month at Sheffield and examined separately, with the following results:

		Mean smoke conc.mg./m.^{3}	Benzpyrene conc. μg./100m.^{3}	Benzpyrene in smoke: p.p.m.
3 "Z" days	February, 1948	1·73	32·8	190
25 other days	February, 1948	0·40	7·2	180
All 28 days	February, 1948	0·55	9·8	180
February, 1949		0·44	6·4	150

The mean concentration during the three "Z" days is more than four times that during the remainder of the month, whilst the relatively small rise in its proportion in the smoke shows that the change is merely one of quantity, and not of quality.

EXPLANATION OF PLATES.

FIG. 1.—Fluorescence spectra of eluates from smoke extracts.
FIG. 2.—Fluorescence spectra. Estimation of benzpyrene in smoke extracts (Sheffield).
FIG. 6.—Fluorescence spectra of eluates from extracts of city smoke.
FIG. 7.—Fluorescence spectra of eluates from extract of car exhaust soot.
FIG. 8.—Fluorescence spectra. Separation of benzpyrene from car exhaust soot.

Burnley Summer 1948
30%A
30%B
50%
Burnley Winter 1948–49
30%A
30%B
50%
Burnley Summer 1949
30%A
30%B
50%
Burnley Winter 1949–50
30%A
30%B
50%
Burnley Summer 1950
30%A
30%B
50%
Bp standard
Hg line
4272
4032
Burnley Winter 1950–51
30%A
30%B
50%
Beckton Summer 1949
30%A
30%B
50%
Beckton Winter 1949–50
30%A
30%B
50%
Beckton Summer 1950
30%A
30%B
50%
Beckton Winter 1950–51
30%A
30%B
50%
Feb(Y) 1948
Feb(Z) 1948
May and June 1948
Dilutions of standard solution
1·0
0·8
0·6
0·4
0·2
July 1948
Aug. 1948
Sept. 1948

Fig. 1

Fig. 2

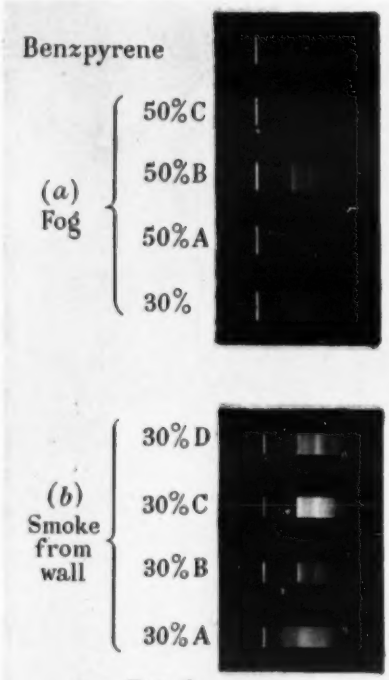

Fig. 6

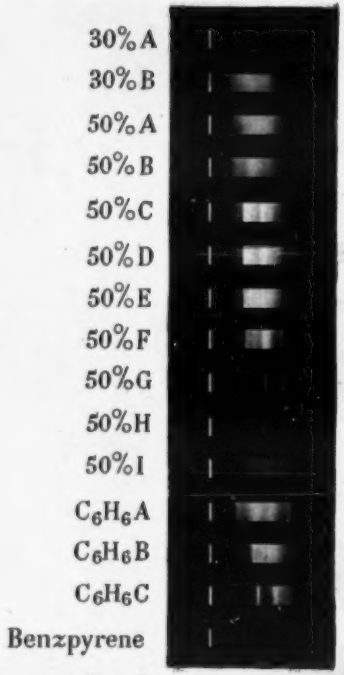

Fig. 7

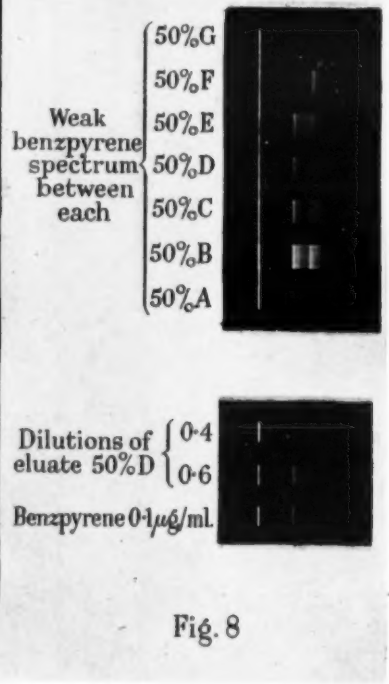

Fig. 8

Foggy conditions allow of the rapid collection of detectable amounts of benzpyrene, which has been shown in this laboratory. By drawing air for 4 hours through a Soxhlet thimble exposed on the roof during fog, a large deposit of black smoke was obtained, which was immediately extracted and analysed by the method adopted for normal smoke filters. The fluorescence spectra of a series of chromatographic fractions of this extract show the spectrum of benzpyrene clearly in eluate 50% B (Fig. 6*a*).

SUGGESTED MODIFICATIONS IN METHOD.

For the more accurate estimation of benzpyrene and more particularly for the determination of its proportion in smoke from different sources, something other than a collection of daily smoke filters is required, and a few small scale trials have been made of other methods. The limitation of an ordinary filter-paper is that it soon becomes blocked with smoke, and must be changed at intervals. The Soxhlet thimble mentioned above, when used in series with a normal filter-paper, forms quite an efficient smoke collector, and will retain very much more smoke than a filter-paper alone. However, a more compact filter which serves the same purpose can be made from a Pyrene filter disc (type 2130) backed with an ordinary filter-paper. Such filters required prolonged washing with acetone in a Soxhlet apparatus before use for benzpyrene work. They can be used for collecting smoke at a low rate over a long period without being changed, but are not suitable where it is required to weigh the smoke collected, as fibres frequently drop out of the filter during use. The use of filter-papers of various kinds has been discussed by Bourne and Street (1950), and Silverman and Viles (1948) have described an apparatus using pleated filter-papers. Filters consisting of salicylic acid crystals lightly packed in a brass tube have also been used, but again difficulties arise in weighing the smoke, as the usual method of volatilising the acid at about 105° C. and weighing the residue was found to drive off some of the benzpyrene, whilst the use of solvents to dissolve out the tarry matter and/or the acid itself does not permit of the weighing of the smoke together with its tar content. For the determination of mean concentrations of benzpyrene over long periods where both the volume of air passed and the weight of smoke collected are required, the solution would seem to lie in a weighable filter prepared from asbestos fibres packed in an aluminium tube (as suggested in Leicester, 'Atmospheric Pollution,' 1945, p. 16). A description of various air-sampling devices used in industry has been given by Silverman (1948).

For rapid determinations over a short period some form of impactor is useful (May, 1945 ; Sonkin, 1946), as the smoke can be collected free from all other material on glass slides. Samples of smoke weighing a few milligrams were collected in a crude single-jet impactor, but as the smoke began to pile up, so the efficiency of the system dropped, thus limiting its capacity. From a 2 mg. sample of smoke obtained in this manner a solution containing 1·7 μg. of benzpyrene was obtained after the usual extraction procedure, representing a concentration of 5μg./100m.^{3} in the air sampled, and 800 parts per million in the smoke collected. This "snap" sample taken on the laboratory roof could not be representative, but it will be seen that the results are of the same order of magnitude as the general ones given earlier.

Another approach is to obtain bulk samples of smoke from large scale equip-

ment. Air-conditioning plants in hospitals and public buildings, and filters on air compressors in some industrial concerns, collect much " suspended impurity " from the air, which may be used for benzpyrene determinations, although it is often difficult to free the smoke from the filtering medium in order to weigh it, and in general the volume of air from which it has been drawn is unknown. A large sample was obtained from the compressed air plant at Beckton, and has been used in pilot experiments to determine a suitable method of dealing with bulk material. To extract the tarry matter without the very finely divided smoke particles entering the solution, the sample was contained in a Soxhlet thimble completely surrounded by filter-paper powder inside another thimble. Since the smoke at Beckton is not representative of normal conditions, no final results were obtained from it, as a more normal sample is now available from the Royal Festival Hall, London.

Some suspended matter is removed from the air by a natural thermal precipitator action, in which warm air rising up the side of a building deposits some of its smoke on projections on the wall. A very large deposit has been built up on parts of the exterior walls of this Department over the past 40 years, and a sample of it yielded fractions (30 %B, C D, Fig. 6*b*) containing benzpyrene. Other bands shown in the spectra of eluates 30%C and D will be discussed below.

Further improvement in accuracy might be obtained by a more complete separation of benzpyrene from other fluorescent substances. In view of the very small quantities involved a high recovery is essential, so that most chemical methods are excluded. Only a limited separation can be effected by chromatography on alumina, and other adsorbents, such as preparations of picric acid or trinitrobenzene on alumina and silica gel (Weiss, 1948 ; Godlewicz, 1949), achieve further separation only at the expense of recovery.

OTHER SOURCES OF BENZPYRENE.

Whilst the above results indicate that much of the benzpyrene found in the air is contained in coal smoke, it may not come exclusively from this source. Benzpyrene is found in some mineral oils, and hence one must consider the possible effect of internal combustion engines. A blue haze of extremely fine sooty particles is sometimes emitted from the exhausts of petrol engines, and occasionally a cloud of black smoke from diesel engines. A small deposit of soot often builds up near the end of exhaust pipes, and this is probably similar to the material dispersed into the atmosphere. Samples taken from a motor cycle, two cars and two diesel compressors have all shown the presence of benzpyrene. Its origin is uncertain, for the question of whether or not it is present in the fuel or lubricating oil used depends on their source and the treatments to which they have been subjected. No benzpyrene was found in particular samples of " Pool " petrol, but, after the separation of large quantities of interfering substances, it was detected in a sample of lubricating oil. However, conditions in the combustion chamber may be suitable for the production of benzpyrene, so that it might be emitted in any case. The soot consists of extremely fine particles, and contains a mixture of hydrocarbons which appears to be even more complex than that in coal-smoke. A large number of fractions showing different fluorescence spectra (Fig. 7) were obtained from a sample of car exhaust soot. Eluates 50% C, D and E show the spectrum of benzpyrene together with that of another substance over-

lapping it. A second chromatogram (Fig. 8) partially separated the two components. Eluate 50% D, as compared with a standard, proved without doubt to contain benzpyrene, whilst 50% F contained an unknown substance with a spectrum similar in form to that of benzpyrene, but shifted by about 250A to the long wave-length side. This substance was of interest since it could also be separated, with difficulty, from certain smoke extracts. A trace of it can be seen along with benzpyrene in eluates 30% C and D (Fig. 6*b*) of the wall smoke extract, and there are suggestions of it in many of the extracts from smoke filters. As an attempt to identify it in domestic soot proved unsuccessful, this may indicate that a small proportion of smoke collected in towns comes from internal combustion engines. It may also be of importance to note that whilst the particles in coal smoke are distributed over a large range of sizes, those in the exhaust of internal combustion engines are concentrated in a limited range of small sizes, and that this may be quite near to the range which undergoes maximum retention in the lung (Wilson and Lamer, 1948; Landahl and Herrmann, 1948; Hatch and Hemeon, 1948; Davies, 1949; Owens, 1923; Boyland, Gaddum and MacDonald, 1947). Hence any further inquiry into this subject might benefit from the segregation of smoke into different size-ranges prior to the estimation of benzpyrene. For this purpose a large capacity cascade impactor would provide a suitable means of collecting the smoke.

Amongst miscellaneous substances tested for benzpyrene was a sample of the "coke" obtained as a by-product in the refining of petroleum. This showed a trace of benzpyrene which, if not present in the crude oil, may have arisen in a cracking process. The benzpyrene found in certain rubbers (Falk, Steiner, Goldfein, Breslow and Hykes, 1951) is attributed to the carbon black used as a filler.

The literature on the carcinogenic action of tobacco tars and smoke contains somewhat conflicting reports, and has been summarised by Flory (1941). Some of the apparent differences may be due to differing conditions of combustion and distillation; only those simulating natural smoking are of any practical interest. In view of the influence of cigarette smoking upon the incidence of lung cancer (Wynder and Graham, 1950; Doll and Hill, 1950) a few experiments were carried out on cigarette smoke, with negative results. However, one must emphasise that it was very difficult to free cigarette smoke extracts from relatively large amounts of a yellow substance soluble in petroleum ether and strongly absorbent to ultra-violet light, so that benzpyrene may possibly have been missed in these experiments, which were carried out partly on cigarette ends collected from several smokers who had avoided any contamination of them, and partly on smoke collected by a filter of salicylic acid crystals loosely packed between cotton-wool in a short glass cigarette holder. The former method represented normal smoking, and assumed that some of the smoke which had passed through the end would have been retained there (Mulinos and Cockrill, 1938). The latter method was not far removed from normal smoking, as the filter offered a low resistance to the passage of air, though, since it removed something like 80 to 100 per cent of the smoke, the smoker considered it far from normal. Cigarette ends were extracted with a large variety of solvents, and salicylic acid filters with petroleum ether alone, which allowed of the ready removal of any dissolved acid by passage through a column of alumina, and elution of everything else as usual, leaving the salicylic acid on the column. Although a large number of blue fluorescent fractions were obtained from each extract, none of them showed benzpyrene.

FATE OF BENZPYRENE IN THE LUNG.

In the large amounts of smoke which we inspire, the larger particles are arrested before they reach the lung, the smallest are breathed in and out without retention, and somewhere in between lies a range of particles which are retained to a limited extent. If some of the benzpyrene in town air is contained in particles within that range, it will gradually be deposited in the lungs of a town-dweller, but whether it stays there unchanged or not is another matter. Berenblum, Crowfoot, Holiday and Schoental (1943), Berenblum and Schoental (1946) and Weigert (1948) have reported the conversion of benzpyrene in the animal body into a number of different products, and possibly benzpyrene in the lung may be so changed. The oxidation of benzpyrene *in vitro* has also been reported by several workers (Warren, 1943; Calcutt, 1944; Weil-Malherbe, 1944; Allsopp and Szigeti, 1946; Cook and Schoental, 1950), and water-soluble products have been found. In this laboratory the products of oxidation of benzpyrene have been separated by chromatography on alumina, and in each case a green-fluorescent substance was eluted from the top of the column with dilute alkali after all other products had been removed; in alkaline solution the brilliant green fluorescence persisted indefinitely, but disappeared on acidification, and if the solution was shaken with benzene a pale blue fluorescence appeared in the latter. Similar substances were detected in aqueous extracts of soot and cigarette smoke and mixtures of the two, but in amounts too small for identification. The possibility of the interaction of certain constituents in coal smoke and cigarette smoke, or of the presence of a water-soluble carcinogen in cigarette smoke itself, should not be overlooked, but this question was outside the scope of the present inquiry.

SUMMARY.

Benzpyrene has been detected in samples of smoke drawn from the air at eight different towns in England. The concentration rises sharply during the winter, and there is a tendency for the mean annual values to increase with the size of the town. A large part seems to come from domestic fires, but it has been detected also in the exhaust from internal combustion engines. None has been found in cigarette smoke.

This investigation has been rendered possible only by the enthusiastic co-operation of a number of individuals, including the following and their respective staffs: Dr. A. R. Meetham, formerly Superintendent of Observations of Atmospheric Pollution at the Fuel Research Station, Greenwich, and his successor Mr. S. H. Richards; the Chief Chemist, London County Council; the Public Analysts of the cities of Bristol, Leicester and Sheffield; the City Analysts of Liverpool, Manchester and Kingston-upon-Hull; the Borough Analyst of Burnley; and the Chief Sanitary Inspectors of the Borough of Bilston, and of the Urban District of Cannock.

The work was carried out during the tenure of a grant from the Medical Research Council. Grateful thanks are due to Sir Ernest Kennaway for his constant advice and encouragement.

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RADIOACTIVE MATERIAL IN THE ATMOSPHERE.

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RADIUM and thorium are widely distributed throughout the earth's crust, although, as yet, no large deposits have been found (it has been estimated that uranium and thorium exist in as much as 4 and 12 parts per million respectively). Both these materials decay through a radioactive series, and in each case one member of the series is gaseous. These two radioactive gases escape into the atmosphere where their respective decay products will be formed and these products will probably attach themselves to dust particles. There are other sources of naturally occurring radioactive material (actinium, which decays to gaseous actinon ; potassium ; rubidium and carbon 14), but these are insignificant compared to members of the radium and thorium series.

The presence of radioactive material in the atmosphere was first observed by Elster and Geitel (1901). Their observations were confirmed by other workers in various parts of the world. Most of these early determinations were made by the charged wire method, which gave quite good qualitative results. An improvement was made when A. S. Eve (1907), following a suggestion of Rutherford, absorbed radon on activated coconut charcoal, the apparatus being calibrated by radon from a measured source of radium bromide.

METHOD.

The method used was to draw air at a constant rate through a filter-paper, and then to measure the deposited activity under an end-window Geiger counter. The filter-paper was Whatman No. 41, and the filter-holder had a 1-inch-diameter circular aperture. The rate of air flow depended on the length of tubing connecting the pump and filter-holder, but was always between 30 and 35 litres a minute. It was measured by a U-tube manometer, and controlled by a screw clip. The manometer was calibrated by a dry gas meter, the accuracy of which was determined for us by Mr. Redding of the British Scientific Instrument Research Association. The sample was placed at once in a brass tray under a General Electric Co. type EHM 2 Geiger counter, housed in a lead castle, and a decay-curve was taken over a period of half an hour. The collecting time was 20 minutes, which does not necessarily give the maximum measurable activity, but allows the experiment to be completed in an hour.

The efficiency of the filter was examined in a series of experiments in which one, two or three filter-papers of different types were used in series as a backing for the Whatman No. 41 filter-paper, and their activity measured. The Whatman No. 41 was found to be at least 99 per cent efficient. The activity of a uranium oxide source was measured before each sample, and the counting rate adjusted

proportionally so that this standard source would give a constant figure. To make the results comparable, the data were adjusted to a pumping rate of 35 litres a minute. This method was suggested by Dr. T. E. Banks, and the apparatus was assembled by Mr. R. E. Waller of this laboratory.

The experimental difficulties in calibrating the apparatus with a known source of radon were prohibitive; consequently, we decided to use a theoretical decay curve corrected for the efficiency of the Geiger counter in measuring the particles from individual members of the radium and thorium series. The geometrical efficiency of the counter is 15·4 per cent. As it was not possible to obtain standard samples of each member of the radium and thorium series, we determined the efficiency of the counter for measuring the β particles from three radioactive isotopes. These efficiencies were plotted against energy, and the efficiency for the β-emitting members of the radium and thorium series were read from this graph.

The efficiency of the counter for measuring α particles cannot be determined in the same way as for β particles, since an unknown number of α particles will be absorbed in the filter-paper. In order to calculate the α efficiency, a dust sample was taken and counted in the usual way; a piece of aluminium foil (thick enough to absorb all the α particles) was then inserted between the filter-paper and the Geiger counter, and the count was continued. This experiment was repeated for a series of samples. The proportion of α to β particles emitted by the dust was deduced theoretically, and the proportion of α particles which were counted was determined from the drop in counting rate caused by interposing the aluminium foil. The efficiency of the counter for measuring α particles was then deduced from these two figures together with the previously determined efficiency of the counter for β particles. It was assumed that the proportion of α particles absorbed in the filter-paper was the same in every dust sample, and that the counter efficiency was the same for all α emitters. The counter used does not record γ rays.

These counting efficiencies were then applied as corrections to the theoretical decay curve which can then be used in the quantitative estimation of the individual samples and in their analysis to determine the proportion of radium A and thorium A present.

RESULTS.

Samples were taken at six different sites during 1950 (Table II); the individual sites are described below.

Pathology Department at St. Bartholomew's Hospital.

(a) *Roof.*—This site was chosen mainly on the grounds of convenience, but it is in a reasonably exposed position in the centre of the city; it is 78 ft. above ground level, but a series of samples taken at various levels on the fire-escape attached to the building showed no significant variation with height. Dust samples were taken daily from December 1949, to June 1950. These samples showed large day-to-day variations in activity, and when more than one sample was taken in one day, there were often considerable differences. Table I shows the activity of the samples for the months of January and May, whilst Table II shows the monthly means.

The most obvious cause of these variations is the weather. The reports of the Meteorological Office give data for Kew Observatory and London Airport.

The data for Kew were taken unless there was a significant difference between these two sites, in which case that day was ignored or mean values were taken. Atmospheric pressure, temperature, humidity, wind force and direction and precipitation were considered. Activity varied directly with pressure and absolute

TABLE I.—*Results obtained on the Roof of the Pathological Department, St. Bartholomew's Hospital, during January and May*, 1950.

January.			May.		
Day of month.	Mean activity (Curies/metre^3) $\times 10^{12}$.	Number of samples.	Day of month.	Mean activity (Curies/metre^3) $\times 10^{12}$.	Number of samples.
2	80·5	1	2	3·1	2
3	56·2	1	3	28·7	1
4	41·6	1	4	20·5	1
5	39·8	1	5	24·0	2
6	26·5	3	8	85·5	1
7	58·0	1	10	57·4	1
9	381·8	2	11	31·9	2
10	148·9	1	12	48·9	1
11	30·6	2	15	25·9	2
12	53·9	1	16	16·7	1
13	85·5	1	17	15·1	1
16	66·9	1	18	37·2	1
17	42·9	2	22	6·6	1
18	67·5	1	23	13·2	1
19	67·8	1	25	21·4	1
20	55·8	2	26	24·3	2
23	45·1	1	30	21·4	1
24	56·8	1			
25	59·8	1	Mean	28·4	Total 22
26	76·3	2			
27	243·6	2			
30	239·5	3			
31	137·6	2			
Mean	94·1	Total 34			

humidity, and inversely with wind force. Neither rainfall nor temperature had any effect. The figures for wind direction were not sufficiently precise to show any relation to activity, but Satterly (1910), after a fairly exhaustive inquiry, showed that the activity increased, the greater the path of the wind overland. Samples taken here on foggy days were very active, but no more active than on some other days without fog.

(b) *Laboratory.*—During February, March and April, of 1950 dust samples were taken in the laboratory on the fourth floor of the Pathological Department, immediately beneath the collecting site on the roof, which showed corresponding variations, but were generally twice as active, due no doubt to the greater emanating surface of the six walls (the parent radium is found in most materials and can often be detected in brick dust) and to the lack of air currents.

Rothamsted Experimental Station, Hertfordshire.

Through the kindness of the Director of the Rothamsted Experimental Station samples were taken during June and July at the meteorological enclosure, which is in an exposed site at the top of a small hill in a semi-rural area, about 30 miles from London. These showed a lower average, and much less variation than those at the Hospital; those taken in the afternoon were usually less active

TABLE II.—*Summary of Results at all Collecting Sites.*

The monthly mean is calculated from the mean daily samples.

Site.	Month, 1949–50.	No. of days.	No. of samples.	Mean activity (Curies/metre^3) $\times 10^{12}$.	Standard deviation.	Highest activity.	Lowest activity.	Activity: highest divided by lowest.	Ratio: Laboratory to roof.
Pathological Department, St. Bartholomew's Hospital:									
(*a*) Roof	December	5	6	121·7	56·9	227·2	43·8	5·2	
	January	23	34	94·1	83·8	384·9	22·4	17·2	
	February	18	33	34·6	21·2	121·8	10·1	12·1	
	March	12	15	51·3	45·5	184·9	10·4	17·8	
	April	4	4	42·3	24·6	79·5	11·0	7·2	
	May	17	22	28·4	19·6	85·5	2·9	29·1	
	June	5	8	63	21·2	113·6	24·1	4·7	
	Whole period	84	122	59·2	59·6	384·9	2·9	131·2	
(*b*) Laboratory	February	18	20	76·6	28	116·7	40·1	2·9	2·4
	March	16	19	94	55·6	229	25·7	8·9	1·9
	April	3	3	106·1	33·2	—	—	—	2·0
	May	9	10	41·4	11·5	60·9	26·8	2·3	1·6
	Whole period	46	52	77·7	43·4	229	25·7	8·9	2·0
Rothamsted	July	26	52	34·4	2·5	78·9	7·6	10·4	
Chiswick:									
(*a*) Control site	August	20	34	45·7	39·5	197·8	12·5	16·3	
(*b*) Cellar	„	13	34	779	456	2,282	213	11·0	
Manchester	November	14	43	78·9	32·7	160·9	18·3	8·8	
Central London:									
(*a*) Control site	December	11	11	96·5	17·7	170·4	36·6	4·7	
(*b*) Air raid shelter	„	11	23	11,780	3,870	21,675	5,110	4·2	

than those taken in the morning. Rothamsted is a third-order station of the Meteorological Office and more accurate correlation of weather phenomena and activity were possible; the conclusions drawn from the Hospital results were confirmed. The activity was greater if the ground was dry or drying. A 100-ft. high wooden tower was available, from which samples were taken at 10 ft. intervals. Again, there was no significant variation with height.

Chiswick.

During August and September results were obtained in the garden of a private house beside the River Thames in Chiswick (West London), which were intermediate between those of Rothamsted and the Hospital. In a closed room ventilation by opening all possible doors and windows (about one-twelfth total wall and floor space) lowered the activity by as much as one-half. A series of samples were taken in the coal cellar beneath the house. The floor of the cellar was only 4 ft. below street level; there was some ventilation from a window and the door leading to the main part of the house. The cellar had not been used for several weeks. In order to avoid disturbing the dust, the pump and filter-holder were kept outside the cellar (Table III). On an average the cellar showed 14 times as

TABLE III.—*Comparison of the Cellar and Exposed Control Site at Chiswick.*

Day of the month, August, 1950.	Mean daily activity (Curies/metre³ of air) × 10^{12}.	
	Cellar.	Control site.
14	685	40·1
16	681	18·6
18	435	14·8
21	549	59·7
22	912	170·7
23	1,305	65·0
24	243	22·7
25	228	17·3
28	745	58·0
29	461	19·2
30	688	85·1
31	1,719	98·4
1	1,483	36·3
Mean	779	54·3

much activity as the control site in the garden. Some samples taken in the house showed that the large activity was confined to the cellar. Samples taken after disturbance of the dust in the Chiswick cellar showed no appreciable increase; the variations were similar to those at the control site, and must therefore have been caused by the weather. The coal dust which covered the floor and walls was not active.

Manchester.

The collecting site was a window-ledge 8 ft. above street level on a building belonging to the Medical Officer of Health's Department, in the city centre. Samples taken during November, 1950, were, on average, the highest yet found, except for the samples taken at this Hospital in December and January (Table II). In Manchester atmospheric pollution is greatest in November. The mass

of the dust was determined by Owen's Scale of Shades (not a very accurate method) but there was no statistical correlation between activity and dust concentration.

Central Telegraph Office.

To test results obtained in the Chiswick cellar, samples were taken in an air raid shelter in Newgate Street, in central London, which was put at our disposal by the Central Telegraph Office. The shelter, of which the ceiling was at ground level, was divided into small rooms from which all draughts were excluded by the blast walls. The heating pipes for the whole Post Office block pass through the shelter, and the temperature was constant at 60° F. to within $\frac{1}{2}$° F. The control site was in the open air, immediately above the shelter. The room used for collecting the samples was kept locked, the pump and filter-holder being in an adjoining room. The activity was very much higher than at any of the other sites investigated (Table IV); on average it was approximately 200 times as great as at the Hospital, that is, nearly one-eighth of the accepted tolerance concentration for radon (Table V). Disturbing the dust had no effect on the activity. Dust sweepings from the floor were not active. Large day-to-day variations were observed, but again these are similar to the variations at the control site.

TABLE IV.—*Results for the Air Raid Shelter, Central London.*

Date, December, 1950.	Time.	Activity (Curie/metre³) × 10^{12}.		Ratio of Shelter to Control site
		Shelter.	Control site.	
4	12.45	6,656	88·3	75
4	14.45	5,111	—	58
5	10.00	13,125	170·4	77
5	18.00	12,683	—	74
6	11.10	12,809	106·6	120
6	15.00	10,793	—	101
7	10.45	5,111	36·6	140
7	15.00	5,868	—	160
8	12.00	14,166	41·6	340
8	15.30	13,598	—	326
11	14.30	7,701	97·8	80
11	16.30	6,404	—	65
11	18.30	7,099	—	73
12	10.30	14,840	52·4	284
12	14.00	14,324	—	273
12	16.15	13,724	—	262
13	11.45	18,015	160·9	112
13	14.15	12,683	—	79
13	16.00	14,071	—	87
14	10.00	21,674	163·7	132
14	15.15	15,933	—	97
15	12.00	13,724	50·5	272
18	10.45	11,169	93·4	120
	Mean	11,796	96·5	122

DISCUSSION OF METHODS.

The experimental procedure was not sufficiently accurate to show the presence of thorium in any of the samples from any of the sites investigated owing to the small counting rate which these gave. Wilson (1909) found 4,000 times as much radon as thoron. On this basis the counts due to thoron in our samples would be much less than the standard error of counting.

None of the samples showed the presence of radon. The proportion of radon atoms that will decay whilst passing through the filter-paper is negligible, but when one considers the very large absorptive capacity of carbon for radon, it is rather surprising that no radon was found.

We have not investigated the proportion of the other members of the radioactive series that will adhere to dust particles. Carmichael and Tunnicliffe (1948) removed the dust from a closed room by fanning the air across a greasy plate and noted the diminution of activity in an air sample. On introducing smoke there was an immediate increase in activity, showing that in a small room there is never an appreciable quantity of free radioactive atoms—they either become attached to dust or migrate to the walls. To our knowledge, no information as to the size range of these dust particles is available.

The results of some of the earlier observers are shown in Table V.

TABLE V.—*Comparison of Results.*

Place.	Average concentration of radon (Curie/metre^3) $\times 10^{12}$.	Ratio of extreme values, highest divided by lowest	Observer.
Montreal	60	7	Eve (1907).
Chicago	89	4·4	Ashman (1908).
Cambridge	105	10	Satterly (1908).
Joachimstal (Jachymov) (in mine)	3,000,000	—	Quoted by Evans (1950).
Droitwich Spa	13,000	—	?
St. Bartholomew's Hospital, London :			
a. Roof	59	130	Present series.
b. Laboratory	78	9	,, ,,
Rothamsted	34	10	,, ,,
Chiswick :			
a. Garden	46	12	,, ,,
b. Coal cellar	779	11	,, ,,
Manchester	79	9	,, ,,
Central London :			
a. Control site	96	4·5	,, ,,
b. Shelter	11,800	4	,, ,,
Tolerance (maximum concentration)	100,000	—	—

The figure for Droitwich Spa was taken from a brochure issued by the local Council. Our inquiries as to who made the original measurements were not very successful, but it is believed that they were made by Professor H. Ballantyne in 1913. Burke and Nolan (1950) have published some results for the radium A content of the air in Dublin :

1947 { Maximum value $54 \cdot 6 \times 10^{-12}$ curie/metre^3 of air (December).
Minimum value $0 \cdot 3 \times 10^{-12}$ curie/metre^3 of air.

1948 { Maximum night value 125×10^{-12} curie/metre^3 of air (June).
,, day value 69×10^{-12} curie/metre^3 of air (March).
Minimum value of $1 \cdot 1 \times 10^{-12}$ curie/metre^3 of air (February and June).

They say that the "normal" value for any one month was $10 \cdot 24 \times 10^{-12}$

curie/metre^3. This is low compared to most observations, but they quote two similar Austrian results :

V. F. Hess, near Vienna ; $9{\cdot}9 \times 10^{-12}$ curie/metre^3 of air.
E. Schrödinger, at Seeham ; $8{\cdot}9 \times 10^{-12}$ curie/metre^3 of air.

Simpson (1905) made a very exacting survey of the air-borne radioactive material in Lapland in 1904, using the charged wire method (Table VI). The

TABLE VI.—*Results obtained in Lapland in* 1904 (*Simpson*, 1905).

The units of activity are arbitrary.

Month.	Mean.				Mean of period.	Maximum.			
	3–5 a.m.	10–12 a.m.	3–5 p.m.	8.30–10.30 p.m.		3–5 a.m.	10–12 a.m.	3–5 p.m.	8.30–10.30 p.m.
November and December	209	87	88	131	129	396	204	384	432
February	221	72	113	101	127	366	234	342	228
April	87	41	37	55	55	210	120	90	120
May and June	79	35	32	43	47	204	102	78	108
July and August	175	35	32	76	80	207	72	93	198
September	201	81	76	142	123	390	156	122	264
Whole period	162	58	62	92	93	396	234	384	432

units are arbitrary. He compared these results with the weather conditions, and summarized his findings by saying that the activity was proportional to the stagnation of the air. The summer minimum is due to the prevailing winds being off the sea, whilst in winter they are off the land.

DOSAGE.

Exceptionally high concentrations of radon will be found wherever radium is handled in bulk. Read and Mottram (1939) have published the results of some determinations of the radon concentration in the radium laboratories at Mount Vernon Hospital, at The Radium Institute, and in the radium workshops of Messrs. Johnson, Matthey & Co., Ltd. (all in or near London). In the last case a concentration of 22×10^{-7} curie/metre^3 of air was observed.

Evans (1950) has calculated the dose received by Joachimstal (Jachymov) miners in a working day through inhaling radon. He has shown that if A is the concentration of radon in the inhaled air, r the radius of the particular section of bronchus, D the range of the particles in tissue and d the density of tissue, then the effective concentration of radon per gram of tissue in the bronchus is $\left(\frac{r}{2Dd}\right)A$. If d is approximately unity, and D equal to 50μ, then $C = 100rA$ (r being in centimetres), i.e., the tissue dose of radiation delivered to the bronchial epithelium is greatest in the larger bronchi.

Using this equation and taking the mean concentration of the radon in the mines as 3×10^{-6} curies/metre^3 of air, he calculates that the miners receive a dose of 0·1 rep per working day (this only includes the dose received from the α particles ; he does not consider the dose received in non-working hours, nor the dose from the active deposit which may be inhaled as such). Assuming an *RBE* of 5, relative to X-rays, then the dose is 0·5 rem per working day.

Assuming that the usual concentration of radon at this Hospital is 60×10^{-12} curies/metre^3 of air, then by a similar calculation the dose received is 2×10^{-5} rem/day. If the concentration in the Post Office shelter is taken as $12{,}000 \times 10^{-12}$ curie/metre^3, the dose received is $2{\cdot}4 \times 10^{-3}$ rem/day. It is very unlikely that anyone received such a dose in this building when it was in constant use during the war. It was then ventilated by an efficient, electrically operated system of fans and air shafts. It is, however, quite conceivable that there are similar buildings (with similar radon concentrations) used as store rooms, and anybody working in such a building for 8 hours a day and spending the rest of the day in an atmosphere of similar radon concentration as that found at the Hospital would receive a dose of 81×10^{-5} rem/day. The tolerance concentration for radon is 10^{-7} curie/metres^3 of air, which is equivalent to a dose of $3{,}500 \times 10^{-5}$ rem/day. All these doses are those received by the bronchial epithelium through inhaling radon.

DISCUSSION OF RESULTS.

Very little difference was found in the gross amount of radioactive material in the atmosphere at four different parts of the country; St. Bartholomew's Hospital, in the City of London; Rothamsted, a semi-rural area, thirty miles from London; Chiswick, geographically in Middlesex but actually a semi-industrial part of London; and in the centre of Manchester. The results were similar to those of other observers in different parts of the world (Table V). Large day-to-day variations in activity were observed, but these can be explained by variations in the weather—the more stationary the air, the greater the concentration of active material. The slight differences between the four sites mentioned above may be seasonal.

The experimental procedure was not sufficiently accurate to show whether thoron or its decay products were present. Radon, absorbed on dust particles, was not detected. No variation with height up to 100 ft. above ground level was observed.

The results obtained in rooms with varying degrees of ventilation are of interest. In the laboratory there was usually twice the activity found at the open-air site on the roof. At Chiswick, ventilating the room lowered the activity by as much as one-half, whilst in the coal-cellar the activity was 14 times greater than at the site in the garden. In the Post Office air-raid shelter the ventilation was as restricted as any found in habitable buildings; the activity was 120 times as high as at the open-air control site, i.e., nearly one-eighth the tolerance concentration of radon. In every case the large day-to-day variations were similar to those at the control site. These large amounts are due to the greater emanating area in a room (compared with the open air) from which the parent radon can escape, and to the restricted ventilation which will allow the radon concentration to build up. The Chiswick cellar was partly below ground and the Post Office shelter entirely so, but this increase in depth (6 ft. at the most) is almost certainly unimportant. It is hoped, however, to investigate the variation of activity with depth at a future date.

SUMMARY.

Measurements of the radio-activity of the suspended matter in the air have been carried out in London at three sites, in Manchester, and in the country at

Rothamsted. The amounts show no considerable difference between urban and rural districts, but there are large day-to-day variations which in situations out of doors are affected chiefly by the wind; the more stationary the air, the greater the activity. In closed places, e.g., an air-raid shelter underground, the amount may be 100 or more times greater than those in the street outside, but even these are small in comparison with the lowest amounts considered harmful to man.

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THE TREATMENT OF ADVANCED BREAST CANCER BY HORMONE THERAPY.

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THE physiological relationship between the breast and the ovary has long been recognised, and as knowledge of this subject has advanced, it has been applied with increasing benefit to the treatment of advanced carcinoma of the breast.

Since the first suggestion by Schinzinger at the Surgical Congress in Germany in 1889 (Schinzinger, 1889) and the first work by Beatson published in 1896, bilateral oöphorectomy has been tried as an auxiliary method of treatment of breast cancer. It has been found of value in younger women. With the advent of X-rays the same effect was brought about by ovarian irradiation (Foveau de Courmelles, 1926; Ahlbom, 1930).

On the experimental side, the work of Lathrop and Loeb (1916), Loeb (1919) and Murray (1928) demonstrated the part played by the ovary in the production of mammary tumours, while Lacassagne (1932) showed that, in certain circumstances, mammary carcinomata could be induced by the injection of oestrogenic substances.

Interest in the problem was further stimulated by the work of Huggins and Hodges (1941) on the treatment of carcinoma of the prostate by castration and stilboestrol.

It was logical that the next step in the management of advanced breast cancer in women should be the use of androgens. Testosterone propionate was tried with success by Ulrich (1939) and by many later workers.

The treatment of women suffering from breast cancer with one of the oestrogen preparations was apparently illogical, although Zondek (1936) had put forward the suggestion in 1936. He pointed out that such treatment would bring about an inhibition of the gonadotrophic and growth hormones of the anterior pituitary, and that this, in turn, would produce a "medical castration" and possibly growth restraint in the tumour.

The next mention in the literature of work of this nature was a symposium published in June, 1944 (Ellis *et al.*, 1944). The work appears to have been started independently in various centres as a result of the growing interest in the relationship between malignant disease and sex hormones. The results amply proved the value of the oestrogens.

Type of case receiving hormone treatment.

Since 1940 various types of hormone therapy have been used in this Department. Such treatment was given to patients who were beyond the aid of surgery

and radiotherapy or who had recurred following orthodox methods of treatment. In these recurrent cases sufficient time had been allowed to elapse, so that previous treatment did not influence the results obtained from hormone therapy.

The present series of 656 cases includes patients treated by ovarian irradiation, by surgical castration accompanied by the administration of testosterone propionate, by various oestrogen preparations (stilboestrol dipropionate, dienoestrol, triphenylchlorethylene and ethinyl oestradiol), by ethisterone and by testosterone propionate alone. It also includes a group treated by irradiation of the pituitary.

Assessment of response.

Assessment of response to the various methods of treatment was based entirely on the observed regression of tumour tissue, and improvement in general health alone was disregarded because such drugs as stilboestrol and testosterone are known to produce this effect in patients not suffering from malignant disease.

Spontaneous regression of tumours has occasionally been reported (Ewing 1940). They are rare, however, and in over 4000 cases of breast cancer observed in this Department no spontaneous regression has so far been noted.

The survival rate will be mentioned in the groups of cases to be considered and, although it seems that life can be prolonged with hormone treatment, no attempt is made to claim success on this account alone.

The responses were graded as follows :

A. *Good response.*—There was complete disappearance of soft tissue metastases or radiological evidence of new bone formation in skeletal metastases.

B. *Fair response.*—There was regression of soft tissue metastases without complete disappearance, or relief of pain in skeletal metastases.

C. *No response.*—There was steady progression of the disease.

These three groups, A, B and C, applied to the series treated by ovarian or pituitary irradiation. Further types of response were noted where hormones were administered :

D. *Adverse response.*—The course of the disease appeared to be accelerated.

E. *Withdrawal response.*—No response was noted during administration of the hormone, but a beneficial effect was produced after the drug had been stopped. This effect has been noted by others (Farrow, 1944). It may be due to a delayed response and the point would require further investigation.

Patients Treated by Ovarian Irradiation.

Treatment by ovarian irradiation was begun in May, 1940, and the results for all patients treated up to June, 1950, have now been assessed.

The technique of treatment has varied slightly over the years, but the aim was to deliver to the ovaries a dose of about 700 to 800r over a period of one week. Where the patient lived some distance away, ovarian irradiation was carried out in a single treatment.

Women of all ages were treated to determine the effect in the different age-groups. Amongst younger women, the dose was sufficient to bring about a cessation of menstruation in all cases.

From a consideration of Table I it will be seen that about 20 per cent of all patients showed a favourable response.

It will be noted too that the average survival rate after treatment by ovarian irradiation was longer in the groups showing a good response. This has to be interpreted carefully because (*a*) there were many individual exceptions, and (*b*) the natural course of the disease is very variable. The difference between the average survival in groups A and C is so great, however, that even bearing these qualifying facts in mind, there seems to be no doubt that life can be prolonged on occasions by ovarian irradiation.

TABLE I.—*Results following Ovarian Irradiation.*

Total number of cases = 175.

Response.	Numbers.	Average survival/mths.	Average age/yrs.	Percentage premenopausal.
A. Good	17 (9·7%)	36·2	43·1	94·1
B. Fair	19 (10·9%)	19·2	44·1	94·7
C. None	139 (79·4%)	10·1	49·7	48·2
Total	175 (100·0%)			

As a general rule the best response occurred in women who had not reached the menopause. Indeed, if premenopausal patients are considered as a separate group, it is found that approximately 32 per cent showed a response of some degree.

Each case was carefully tabulated according to the response shown and according to the histological type of tumour, but no correlation between the two could be found. All histological types of tumour showed a good response on occasions.

An attempt was also made to correlate the degree of response with the site of the metastases, but, here again, no correlation was found. Good responses were seen in all types of metastases, skin nodules, glands, pulmonary and skeletal metastases. In 3 patients with osteolytic bone metastases there was radiological evidence of new bone formation. In 4 a pleural effusion disappeared and in 4 it diminished in amount. One patient showed regression of orbital metastases.

A short account is given of 2 of the patients showing a good response:

Case No. 26,101, *Mrs. E. T*—.—This patient was first seen on 27.vii.46 when she was 39 years of age and about 4 months pregnant. She had a carcinoma of the left breast and widespread osteolytic skeletal metastases. She was treated by simple mastectomy and palliative X-ray therapy to the region of the acetabula. Two months later (15.ix.46) a film of the pelvis (Fig. 1) showed that there was still very extensive metastatic involvement of the acetabular regions, and on this account a caesarean section was performed on 11.x.46. Fig. 2 shows the appearance of the pelvis on 29.x.46. Following this she had ovarian irradiation but the whole pelvis was not irradiated. New bone formation occurred in parts of the skeleton beyond the treated area. Fig. 3 illustrates the remarkable response that had occurred in the pelvis by 14.iv.47. Her general health improved immensely and she became pain-free, but later her condition deteriorated and she died on 3.vii.47.

Case No. 17,361, *Mrs. C. S*—.—This case illustrates the remarkable reduction that may occur in a pleural effusion. She was originally treated by simple mastectomy and X-ray therapy in 1943 for a Stage I carcinoma breast when

she was 38. In November, 1945, she developed a right-sided pleural effusion as shown in Fig. 4. She received ovarian irradiation and the effusion gradually diminished in amount. Fig. 5 illustrates the findings on 9.x.46. After this her general condition became poorer and she died in February, 1948.

Patients Treated by Surgical Castration and Testosterone.

In 1941 a small group of patients was treated by surgical castration, accompanied by the administration of testosterone. This was done in an attempt to find out why there were some failures with ovarian irradiation. It was thought that ovarian function might recover to some extent after irradiation and that the effect of oöphorectomy would be more lasting.

Nine patients with advanced breast carcinoma were treated in this manner by oöphorectomy, followed by daily intramuscular injections of 25 mg. testosterone propionate for periods up to 3 months.

Only one patient showed a slight response. She was a premenopausal woman of 40 with an inoperable breast carcinoma and bone metastases. The primary tumour became smaller and the glands disappeared temporarily, but no effect was noted on the skeletal metastases.

Patients Treated by Oestrogen Therapy.

In the latter part of 1942 it was decided to investigate the value of oestrogens in advanced breast carcinoma, and since that time the following oestrogens have been tried: stilboestrol dipropionate, dienoestrol, triphenylchlorethylene and ethinyl oestradiol. The drugs were given to women of all ages.

Stilboestrol dipropionate.

The dose used in the majority of patients was 5 mg. twice a day. This level of dosage produced only slight gastro-intestinal upset as a rule, and appeared to be adequate for the production of a good response. In a few patients where a gastro-intestinal upset was troublesome the dose was reduced to 2·5 mg., 1 mg. or even 0·5 mg. twice a day.

In patients with a pleural effusion, 5 mg. twice a day sometimes caused sufficient water retention to increase the pleural effusion and add to the patient's dyspnoea. It was remarkable to find in a few such patients that doses as small as 0·5 mg. twice or thrice a day were effective in causing a marked reduction or even disappearance of the effusion.

The drug was given for an initial period of 6 to 8 weeks and then on alternate months. Administration was continued indefinitely where a good response was obtained, even although in some cases the patient became clinically free from disease. This intermittent form of dosage seemed to prolong the good effect, and also minimised endometrial hyperplasia and the risk of withdrawal menorrhagia. Slight vaginal bleeding or discharge occurred intermittently in most patients.

The incidence of toxic symptoms was not unduly high. The number of patients having no symptoms attributable to the hormone while taking stilboestrol was 122, while many of the remainder had symptoms which did not inconvenience them greatly, the most common being nausea and vomiting at the commencement

of treatment. Many complained of frequency of micturition and incontinence. Only 8 showed complete intolerance of the drug when given at a lower dosage.

It can be seen from a consideration of Table II that there was quite a gratifying response—approximately 30 per cent showing some degree of improvement.

The relation between response and the time of occurrence of the menopause is as striking as in the group treated by ovarian irradiation, only the reverse obtains here—the best results are seen after the menopause. There is also an exceptionally high proportion of adverse responses among premenopausal women. This is not a rigid rule, however, as there were adverse responses in older women (the oldest was over 70) and there were fair responses in young women.

TABLE II.—*Results following Treatment with Stilboestrol Dipropionate.*

Total number of cases = 322.

Response.	Numbers.	Average survival/mths.	Average age/yrs.	Percentage premenopausal.
A. Good	33 (10·3%)	30·5	66·3	0
B. Fair	65 (20·2%)	19·9	62·9	9·2
C. None	183 (56·8%)	9·1	57·9	17·5
D. Adverse	33 (10·3%)	6·3	54·7	33·3
E. Withdrawal	8 (2·4%)	27·3	58·1	12·5
Total	322 (100·0%)			

Again, as a general rule, those who showed a good response survived longer, although there were several notable exceptions.

As in the group treated by ovarian irradiation, no correlation was found here between response and the histological type of the tumour.

In general, where a good response occurred, all metastases showed improvement. However, in some patients one metastasis might regress while another became larger, or new ones appeared.

All types of metastases were found to respond on occasions—skin nodules, glands, lung and orbital metastases. Even pleural effusions dimished in amount, and in 3 cases disappeared altogether with no treatment other than stilboestrol dipropionate. It is also very interesting to note that in this series 13 patients with bone metastases experienced relief from pain, while in 6, new bone formation was demonstrated radiologically in osteolytic lesions. As this is not a finding generally reported it may be useful to illustrate it with the following case:

Case No. 11,124, *Mrs. E. B*—.—This patient had a radical excision of the right breast for carcinoma in 1928 when she was 43. She kept well until October, 1944, when she developed proptosis of the left eye. She was seen in the Radiotherapy Department for the first time on 24.v.45, when she was found to have metastases in the left orbit, enlarged glands in the left side of the neck and diffuse skeletal metastases. Fig. 6 shows the presence of osteolytic and osteoplastic metastases in the pelvis on 24.v.45.

Stilboestrol dipropionate, 5 mg. twice a day, was prescribed, and by 2.viii.45 the proptosis had almost gone and the glands were no longer palpable. The left eye appeared normal apart from slight ptosis of the upper lid. Fig. 7 shows the radiographic appearance of the pelvis on 19.viii.47. There had been considerable improvement, and some of the metastases were difficult to detect.

She continued to take stilboestrol dipropionate 5 mg. twice a day on alternate months and remained well until December, 1948. She developed a tumour of the cardiac end of the stomach and died on 27.viii.49. No post-mortem was carried out, but this gastric tumour was probably metastatic in nature from the carcinoma breast.

The following case illustrates the improvement which may take place in a patient with a pleural effusion:

Case No. 127/48, *Mrs. J. L*—.—This patient was treated by simple mastectomy and X-ray therapy in January, 1948, for a Stage I carcinoma of the right breast when she was aged 64. She remained well until November, 1948, when she developed a metastasis in the opposite left breast, which was treated by simple mastectomy and a palliative course of X-ray therapy.

On 19.i.50 she was found to have a right-sided pleural effusion (Fig. 8) extending to the level of the third rib anteriorly. Treatment with stilboestrol dipropionate 0·5 mg. thrice daily was commenced, and by 24.ii.50 there was already considerable reduction in the pleural effusion (Fig. 9). Further reduction took place, until by June, 1950, the findings were within normal limits (Fig. 10). She remained well until October, 1951, when she developed a febrile illness, and an X-ray of her chest showed diffuse pulmonary metastases. Her general condition is now deteriorating rapidly (December, 1951).

Dienoestrol.

The dosage of dienoestrol used was 1 mg. thrice daily in most cases. A few received 5 mg. twice a day with no apparent advantage. The incidence of toxic symptoms was minimal. Where a good response occurred the drug was continued indefinitely on alternate months.

The responses are shown in Table III.

TABLE III.—*Results following Treatment with Dienoestrol.*

Total number of cases = 32.

Response.	Numbers.	Average survival/mths.	Average age/yrs.	Percentage premenopausal.
A. Good	2 (6·3%)	44·0	67·0	0
B. Fair	3 (9·4%)	9·0	60·5	0
C. None	23 (71·8%)	17·6	59·0	0
D. Adverse	4 (12·5%)	9·5	45·8	75
E. Withdrawal	0	—	—	—
Total	32 (100·0%)			

The results shown in Table III are similar to those obtained in the group treated with stilboestrol in respect of age distribution and relation to the menopause. The numbers treated with dienoestrol are smaller, but a comparison of Tables II and III would tend to suggest that dienoestrol is less effective than stilboestrol.

Of those who did not respond well, one showed a very good and 4 a fair response to stilboestrol.

One patient aged 64 who did not respond to dienoestrol showed a fair response to testosterone. This would seem to suggest that a patient may respond to one

type of hormone therapy and not to another, and it would be of interest to pursue this point further.

Triphenylchlorethylene.

Only 6 cases were treated with triphenylchlorethylene. The dosage given was 0·05 mg. twice a day. No case was made worse, but only one patient, aged 79, showed a slight response. Glands were reduced in size and lung metastases did not advance for 4 months.

Ethinyl oestradiol.

The dosage of ethinyl oestradiol used in the majority of patients was 0·05 mg. twice a day. Out of 38 patients treated, 12 experienced a considerable degree of nausea and vomiting, especially in the first week of treatment. Some complained also of palpitation and vertigo. In 2, the dose had to be reduced to 0·05 mg. a day and, in other 2, it had to be reduced to 0·01 mg. twice a day. This drug has only been used since December, 1948, and it is too soon to assess survival rates.

TABLE IV.—*Results following Treatment with Ethinyl Oestradiol.*

Total number of cases = 38.

Response.	Numbers.	Average age/yrs.	Numbers who were premenopausal.
A. Good	2 (5·3%)	65·0	0
B. Fair	10 (26·3%)	61·0	1
C. None	24 (63·2%)	61·5	1
D. Adverse	1 (2·6%)	44·0	0
E. Withdrawal	1 (2·6%)	53·0	1
Total	38 (100·0%)		

Here again the results obtained are comparable with those shown in Tables II and III.

The general impression gained from a study of Tables II, III and IV is that stilboestrol dipropionate is the oestrogen preparation most likely to give beneficial results. Dienoestrol, however, might be tried in a patient who is markedly upset by stilboestrol.

Patients Treated by Testosterone.

A further series of 30 cases was treated with testosterone between July and December, 1949.

The dose used was 50 mg. of testosterone propionate daily, given intramuscularly. Treatment was continued for 3 to 4 weeks, and if the patient's condition showed some improvement, further treatment was given either by means of an implant of 400 to 600 mg. of testosterone propionate into the abdominal wall or by means of methyl testosterone. Methyl testosterone was given at a dose-rate of 50 mg. a day on alternate months, and was administered in the form of tablets which were allowed to dissolve sublingually.

The results are as shown in Table V.

TABLE V.—*Results following Treatment with Testosterone Propionate.*

Total number of cases = 30.

Response.	Numbers.	Average age (years).
A. Good	1 (3·3%)	50
B. Fair	7 (23·4%)	48·2
C. None	20 (66·7%)	45·3
D. Adverse	1 (3·3%)	43
E. Withdrawal	1 (3·3%)	52·1
Total	30 (100·0%)	

A remarkable improvement occurred in the general health of about 50 per cent of the patients treated with testosterone, and this feeling of well-being may not be undesirable, especially in the advanced stages of breast carcinoma. It must be emphasised, however, that improvement in general condition was not taken as a criterion of response, and only patients in whom the tumour regressed have been included among those showing favourable responses.

In this group of cases there seems to be no correlation between the type of the response and the age of the patient. Favourable responses occurred in pre- and post-menopausal women, and also in some premenopausal patients who had failed to respond to ovarian irradiation.

The best results in this group occurred in patients with soft tissue metastases. In one, enlarged glands disappeared completely, and in another 2 the primary tumour showed a temporary regression. One patient who was comatose with cerebral metastases improved sufficiently under treatment with testosterone to attempt crossword puzzles. Subsequent post-mortem examination confirmed the presence of large intracerebral metastatic deposits, which presumably had regressed only temporarily under the influence of testosterone.

In another patient a large pleural effusion did not cause any further respiratory embarrassment, and required aspiration only at increasingly longer intervals. This patient is still alive 25 months after the commencement of treatment.

Only one patient experienced relief of pain from skeletal metastases while receiving testosterone. Another patient with metastases in bone stated she felt the pain easier when the drug was withdrawn. There was no radiological evidence of repair occurring in bone metastases. The series, although small, is in this respect at variance with the results so frequently reported in the literature (Adair *et al.*, 1949; Kaae, 1949; Report of the Council on Pharmacy and Chemistry, 1949).

Only one patient could not tolerate the drug. She was a woman of 43 with cerebral metastases, who developed very severe vomiting, and treatment had to be discontinued after she had received 150 mg. Apart from this one patient no one was upset by treatment. With the dosage used a few complained of nausea at the beginning, but this passed off. Signs of masculinisation were seen in most patients who received 2000 mg. or over. One patient developed a mild acne, which disappeared when the testosterone was stopped. Premenopausal patients developed amenorrhoea.

Patients Treated by Ethisterone.

In order to make the investigation more complete it was felt that the effect of ethisterone (androhydroxyprogesterone) should be tried.

Accordingly it was given to women (*a*) who had not yet reached the menopause, as it was thought that they might show an adverse response to stilboestrol, and (*b*) who had already shown an adverse response to stilboestrol. Actually, only 7 were treated with this drug, but none showed any response.

Patients Treated by Pituitary Irradiation.

In October, 1949, an investigation was begun into the effects of irradiation of the pituitary on the course of breast cancer. It was thought that the treatment would bring about a complete cessation of all oestrogenic activity by suppression of the gonadotrophic hormones. It was hoped that this would produce better results than ovarian irradiation in younger women. Treatment was also given to post-menopausal women to determine the effect.

Many of these patients had already failed to respond to other methods of hormone therapy, and they were beyond the aid of all other forms of treatment.

The pituitary was irradiated by two opposed fields, each 6 × 8 cm. placed in the temporal region, and a dose of 3000r was delivered in 3 weeks to the region of the sella turcica.

A series of 37 patients was treated between October, 1949, and October, 1950, and the results are shown in Table VI.

As will be seen from Table VI, only 2 patients showed a good response. The first patient was a woman of 51 whose periods had stopped 5 months before and who had had no previous hormone treatment. A recurrent mass over the sternum, 6 cm. in diameter, disappeared during the 3 weeks' treatment to the

EXPLANATION OF PLATES.

Fig. 1.—Case No. 26,101, Mrs. E. T— (15.ix.46).—Radiograph of pelvis before ovarian irradiation showing extensive osteolytic metastatic involvement, particularly in region of acetabula.

Fig. 2.—Same case as Fig. 1 (29.x.46).—Radiograph of pelvis following caesarean section but before ovarian irradiation was carried out.

Fig. 3.—Same case as Fig. 1 (14.iv.47).—Radiograph of pelvis 5 months after ovarian irradiation showing new bone formation in acetabular regions and rings of sclerosis round osteolytic lesions in ilii.

Fig. 4.—Case No. 17,361, Mrs. C. S— (13.xi.45).—Radiograph of chest showing pleural effusion on right side (before ovarian irradiation).

Fig. 5.—Same case as Fig. 4 (9.x.46).—Radiograph of chest 11 months after ovarian irradiation showing considerable diminution in the amount of the pleural effusion with the presence of a small fibrin body in the right costo-phrenic angle.

Fig. 6.—Case No. 11,124, Mrs. E. B— (24.v.45).—Radiograph of pelvis showing widespread osteolytic metastatic involvement (before administration of stilboestrol).

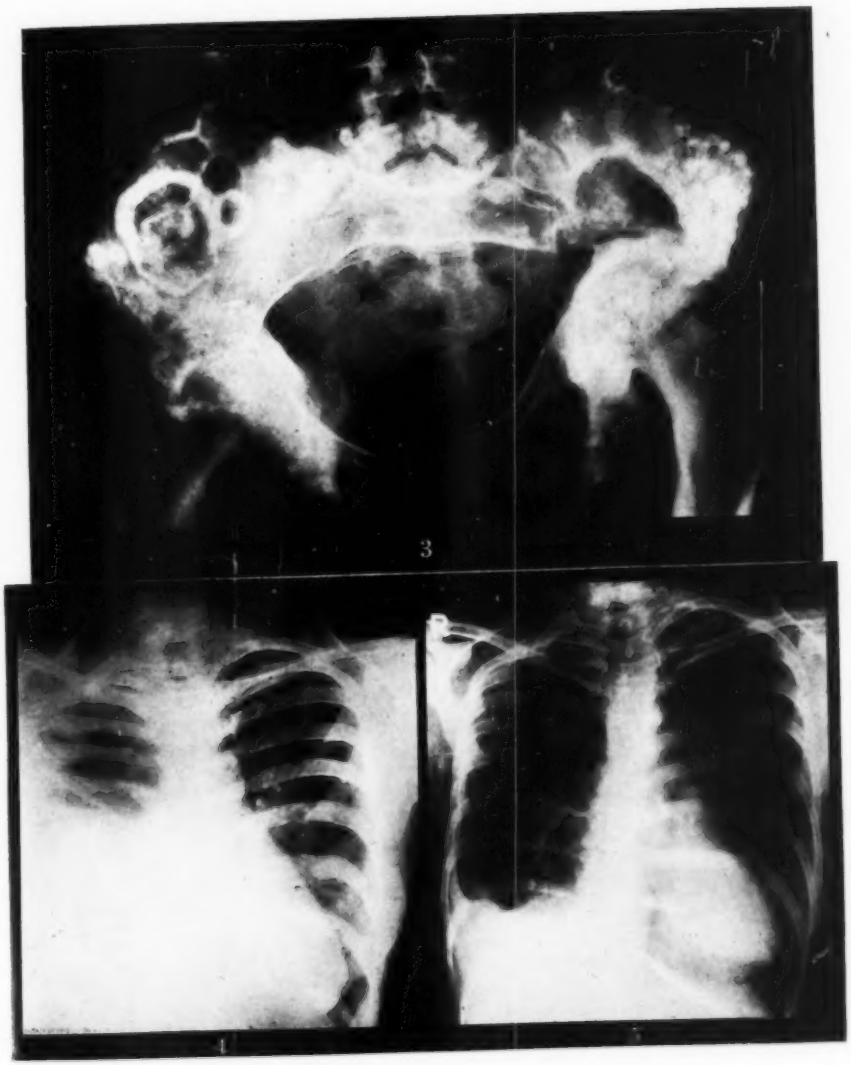
Fig. 7.—Same case as Fig. 6 (19.viii.47).—Radiograph of pelvis 25 months after commencement of treatment with stilboestrol. There has been considerable new bone formation and restoration of the normal trabecular pattern.

Fig. 8.—Case No. 127/48, Mrs. J. L— (19.i.50).—Radiograph of chest showing large pleural effusion on right side (before treatment with stilboestrol).

Fig. 9.—Same case as Fig. 8 (24.ii.50).—Radiograph of chest 1 month after commencement of treatment with stilboestrol. There has been some diminution in amount of the pleural effusion.

Fig. 10.—Same case as Fig. 8 (16.vi.50).—Radiograph of chest showing complete disappearance of pleural effusion.

Douglas.



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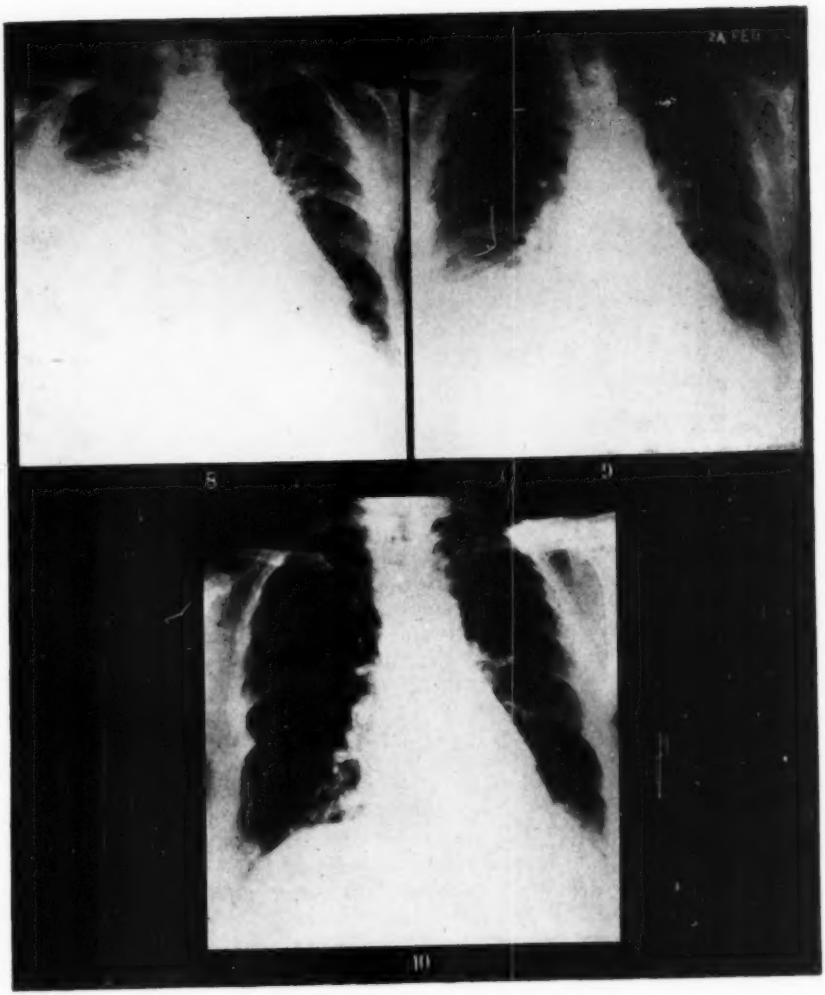

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TABLE VI.—*Results following Pituitary Irradiation.*

Total number of cases = 37.

Response.	Numbers.	Average age (yrs).
A. Good	2 (5·4%)	48·5
B. Fair	2 (5·4%)	54·0
C. None	33 (89·2%)	52·9
Total	37 (100·0%)	

pituitary in October, 1950. She remained well until August, 1951, when she developed skeletal metastases, and her general condition is now very poor (December, 1951).

The second good response occurred in a woman of 46. She had had an artificial menopause brought about by ovarian irradiation one year previously in an attempt to control a local recurrence of the tumour. The recurrence had become flatter for 3 months, but after that had spread again. Following pituitary irradiation complete healing took place slowly, but new nodules appeared 10 months later.

Of the 2 women showing a fair response, the first was aged 46 and premenopausal. She had a skin recurrence on the chest wall within the irradiated area and was treated by pituitary irradiation in December, 1949. Slight regression of the recurrence was noted for 3 months, but then the disease became widespread and she died on 6.xii.50. She had no periods following pituitary irradiation. The other patient showing a fair response was aged 62 and had had a normal menopause 7 years previously. In December, 1949, she developed oedema and congestion of the face and neck due to pressure from enlarged mediastinal glands. She was treated with pituitary irradiation and her symptoms were relieved during treatment. Unfortunately, one month later she developed other metastases and her condition deteriorated. She died on 6.vi.50.

Among the 33 who did not respond the ages varied from 30 to 73. Twenty-two had had ovarian irradiation previously, 6 had had stilboestrol and 2 testosterone. None had shown any response to the other types of hormone treatment. It would seem from this that pituitary irradiation may be of value in the type of case suitable for ovarian irradiation, or in cases where ovarian irradiation has already been given with some good temporary effect.

There is no doubt that pituitary irradiation was the least well tolerated of all forms of hormone therapy. Fourteen patients were very upset by the treatment before the course was completed. It is possible that a modification of technique and dosage would overcome some of these difficulties.

Of the 37 patients, 21 have died within one year, the average survival being 2 to 9 months after the commencement of treatment. Of the remaining 16, only 5 are now able to be up and about.

Comparison with Cases Reported in the Literature.

The results which have been obtained agree with those already reported in the literature as far as response in relation to age and the date of occurrence of the menopause are concerned. There are, however, several interesting points of difference, and some facts which have not been specially noted before.

In the first place, although many series of cases have been published in the past emphasising the value of ovarian irradiation in the treatment of premenopausal women with advanced breast cancer (Foveau de Courmelles, 1926; Ahlbom, 1930; Dresser, 1936; Martin, 1936; Smith, 1936), this particular method appears to have been neglected in recent years in favour of testosterone. The findings in Edinburgh suggest that it might with advantage be more extensively used. It is very simply given as a single treatment in an X-ray department, whereas treatment with testosterone is prolonged and expensive. It is true that some women have troublesome side-effects such as flushings after ovarian irradiation, but the results of prolonged testosterone administration, hirsutes and other signs of masculinisation are not less distressing.

All writers are agreed on the value of the oestrogens in post-menopausal women (Ellis *et al.*, 1944; Haddow, Watkinson and Paterson, 1944; Taylor, *et al.*, 1948; "Report of the Council on Pharmacy and Chemistry," 1949; Adair, *et al.*, 1949; Walpole and Paterson, 1949). However, most reports state that the best responses occur in patients with soft tissue metastases. It is generally considered that oestrogens are of little value in the treatment of skeletal metastases, although Paterson (1944) reported relief from pain in such cases. In the present series it was found that response was as likely to occur in bone as elsewhere and, indeed, in 6 cases there was radiological proof of repair occurring in osteolytic metastases.

Conversely, our experience here with testosterone has been that almost all the favourable responses have occurred in extra-skeletal metastases. No good results were seen in bone lesions apart from relief of pain in 2 cases, although, of course, the series was small, and treatment may not have been continued over a long enough period. The reports in the literature on the efficacy of testosterone are very variable. All gradations of response have been reported. Farrow (1944) noted a deleterious effect in female patients, Nathanson (1944) observed no effect at all, and various later workers (Adair, 1947; Adair *et al.*, 1949; Kaae, 1949) reported a proportion of good responses in skeletal and extra-skeletal metastases. All are agreed that testosterone may produce a beneficial effect on the general health but this is a non-specific effect, and due partly to the stimulating effect of the hormone on protein metabolism. It is, however, important not to assess improvement in general health as an anti-carcinogenic effect.

With both oestrogen and androgen therapy some responses were noted here after the drug was stopped—a withdrawal response. Farrow (1944) also noted this occasionally in the metastases after withdrawal of either oestrogens or androgens.

Consideration of the Mode of Action of Hormone Therapy.

While hormone therapy is widely employed, the basis of treatment remains largely empirical. The extension of our knowledge of the exact mode of action would be of great interest, and might result in further advances in this form of therapy. The clinical results obtained, however, appear to be due to some modification of the endocrine status of the patient.

For example, in a young patient who has not reached the menopause, oöphorectomy or ovarian irradiation causes a sudden withdrawal of the natural ovarian hormones which influence the rate of growth of the cells of breast tissue whether these cells are normal or malignant. The withdrawal of these naturally occurring

oestrogenic substances results in an increased output of the gonadotrophic hormones of the anterior pituitary which stimulate extra-gonadal sources of oestrogenic activity such as the suprarenal cortex. Thus, as time passes, the hormone balance of the body is partly restored and the beneficial effects of castration wear off.

Pituitary irradiation must produce a similar effect to ovarian irradiation in younger women, and bring about a cessation of all natural oestrogenic function. The temporary effect obtained in the cases treated might, therefore, be due to the level of dosage employed. At this stage of our knowledge, however, it is doubtful if it would be justifiable to use a higher dosage.

The administration of an artificial oestrogen again modifies the endocrine status. The oestrogens may produce a direct effect on the breast cancer cells, or the effect may be obtained indirectly through the anterior pituitary.

If the action is a direct one, the best results would be expected in older women. In an older patient, for example a woman who is 5 or more years past the menopause, there is little or no circulating oestradiol, and the addition of oestrogens produces a change. After the passage of time, however, the body, and in particular the breast cells, may become readjusted, and this might explain why the good effect passes off.

The work of Zondek suggests that the oestrogens may have an indirect effect through the anterior pituitary. Zondek (1936) found that the administration of large doses of oestrin produced a chromophobe adenoma of the pituitary in male rats, that is, in rats not normally subject to the action of large amounts of oestrogen.

Zondek (1947) also reports a case in which he gave large doses of oestradiol benzoate to a woman of 26 with metastases from a carcinoma breast. No effect was noted on the metastases, but after death her pituitary was found to weigh 710 g. as compared with a normal of 595 g. (the average for a nullipara of her age according to Zondek). The increase in size of the pituitary was due to an eosinophil adenoma.

Unless the production of an adenoma of the pituitary interferes with the output of other hormones the explanation is not very satisfactory in older patients.

In a premenopausal patient, a drug such as stilboestrol may cause inhibition of the gonadotrophic hormones, so suppressing natural ovarian secretion, but not bringing about a true castration. Castration could not be said to be complete while an artificial oestrogenic substance is circulating in the blood. Consequently, there is not the same profound change in the endocrine status and no useful therapeutic effect is produced.

The question of the administration of testosterone might now be considered. In a premenopausal patient this brings about an artificial menopause and, from this point of view, testosterone might be expected to produce similar results to ovarian irradiation or oöphorectomy. In addition to this, the administration of testosterone must disturb the normal oestrogen-androgen ratio. Finally, testosterone, like the oestrogens, causes inhibition of the gonadotrophic hormones of the anterior pituitary (Moore and Price, 1932). It may, therefore, like the oestrogens, produce an indirect effect on the breast cells by a disturbance of pituitary function.

An explanation must now be sought for the failures. By any method so far outlined the successes do not constitute more than about 30 per cent of the total. It is possible that some patients have a more stable endocrine system,

due to more rapid metabolic processes which quickly counteract the effects of therapeutically induced changes. In this connection the liver must play an important part, because the liver inactivates ovarian oestrogens (Golden and Severinghaus, 1938). In hepatic cirrhosis there is an excess of free oestrogenic substances in the blood. Westerfeld (1940) says tyrosinase, on incubation, inactivates oestrone, oestradiol and diethylstilboestrol. Zondek and Sklow (1942) believe the inactivation is caused by an enzyme—oestrinase—similar to but not identical with tyrosinase. Jailer (1948) found that in mice fed on a diet deficient in vitamin B_1, the degradation mechanism of oestrogens in the liver was impaired. It was not the vitamin B_1 deficiency but the concomitant inanition which was found to be the cause. In addition to the more common causes of liver dysfunction, patients with carcinoma of the breast may have metastatic involvement of the liver. How this would affect the response is not known exactly, but it is perhaps significant that few patients with clinical evidence of liver involvement showed any response to any of the methods of treatment mentioned.

There may, however, be other factors modifying the response. The type of tumour may play a part. Haddow, Watkinson and Paterson (1944) suggested that the tumour called "carcinoma of the breast" might in reality comprise several categories. Walpole and Paterson (1949) also made the same suggestion, even going so far as to say that a tumour might produce an "anti-hormone" or inhibitory factor antagonising the action of the oestrogen. They supported this theory by their findings that, in general, patients who did not respond to oestrogen therapy did not show such a complete degree of keratinisation of the vaginal epithelium. If this change in the original epithelium is not obtained in a post-menopausal woman, they suggested that some inhibitory factor was at work. It seemed as though some substances were being produced, either in the tumour or elsewhere, which were able to nullify oestrogenic activity.

A great deal of further work will be necessary before the exact mode of action can be determined.

SUMMARY AND CONCLUSIONS.

An extensive series of cases of advanced breast carcinoma treated by hormone therapy has been surveyed and the results obtained have been presented.

The findings suggest that, in premenopausal women, the treatment of choice is ovarian irradiation, which was found to produce beneficial results in 30 per cent of patients in this category.

In women who are 5 or more years past the menopause, oestrogen therapy would appear to be the most satisfactory form of treatment. The drug found to be most generally useful was stilboestrol dipropionate 5 mg. twice a day on alternate months.

While the results of treatment with testosterone have been disappointing, it would appear to be the most appropriate method of treatment for post-menopausal women who are not more than 5 years past the menopause, and it should be noted that responses can be obtained where the metastases are confined to soft tissues.

Pituitary irradiation is no longer used routinely, because of the marked systemic disturbance produced. It may be of value in cases treated by ovarian irradiation where the good effect has passed off.

It should be noted that both oestrogens and androgens promote water retention, and in a patient with a pleural effusion the dose has to be greatly reduced.

Good results were noted with doses of stilboestrol dipropionate as low as 0·5 mg. twice a day.

While ordinary methods of histological examination have failed to show any correlation between histological type of the tumour and the response to hormone therapy, it is possible that there are some differences in the cell which are not detected by present methods.

Several suggestions have been offered to explain the mode of action, but much work requires to be done on this aspect of the subject before further advances on the therapeutic side are possible.

In conclusion, I would like to express my thanks to Professor Robert McWhirter for his help and advice in the treatment of these cases and in the preparation of this paper.

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THE GRADING OF OSTEOGENIC SARCOMA.

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DURING the past thirty years much useful progress has been made in the histological grading of malignant tumours of epithelial origin. This work, of which one practical application has been the attempted correlation of prognosis with histological structure of a tumour, has had many valuable results; and although there may be not infrequent discrepancies between the histological appreciation and the outcome of treatment in any given type of neoplasm, the results have at any rate led to some attempt being made by the histo-pathologist to guide the clinician at least in qualitative terms of high or low malignancy; and to more precise premises for the comparison of differing methods of treatment in series of comparable cases.

The starting point of this present analysis was the observation that irrespective of the mode of treatment applied, the overall five year survival rates in most published dissections of groups of cases of osteogenic sarcomata were approximately of a similar order. A recent exception to this is contained in the analysis of a group of 415 malignant tumours involving bone published by Prevo (1950). This large series included a group of 41 cases of osteogenic sarcoma confirmed by biopsy in which there were two 5-year survivals (4·9 per cent). The mean duration of symptoms prior to treatment in this series was 8·6 months, which is rather prolonged. In the small group in the Bristol Bone Tumour Register the mean time from the onset of symptoms to the time of clinical and radiological diagnosis was 4·4 months, and in the British Empire Cancer Campaign analysis (1949) it was shown that 69·9 per cent of all cases attended for their first consultation in 1 to 3 months from the time of onset of symptoms. In Prevo's (1950) series the average duration of symptoms to the time of death was 22·6 months, in the Bristol Bone Tumour Register group here reported this period was rather shorter—18·1 months.

Prevo (1950) comments that the 5-year survival rate in his series does not agree with those published by Geschickter and Copeland (1949) and others, but makes no suggestion to account for the marked discrepancy.

Although the number of cases of osteogenic sarcoma available in the Bristol Bone Tumour Register collection is still small (36), it will presently be shown that in broad outline the series conforms with the general features more clearly delineated by the examination of much larger numbers of cases. It was therefore thought useful to subject the clinical, radiological and histological records of the 36 cases to a close examination with a view to the establishment of a means of grading these tumours on their histological structure, and to determine to what extent the biological history of each particular tumour could be matched with the microscopic picture.

It is noteworthy that two of the most valuable general works in this field—Geschickter and Copeland's 'Tumours of Bone' (1949) and Coley's ' Neoplasms of Bone ' (1949)—make no mention of histological grading of the tumours except in general terms, and although the latter subdivides cases into those of low, average and high malignancy, there is but little to indicate the histological features by which the differentiation can be made, other than the examination of cell morphology and stromal character. It is not infrequently the fashion to subdivide osteogenic sarcoma into a number of types—medullary and sub-periosteal, telangiectatic, sclerosing, periosteal, fibrosarcoma, chondrosarcoma, etc. This does not always seem profitable, and the writer is in accord with the opinion expressed by Willis (1948) on this point. Chondrosarcoma appears to differ in several major ways from the typical osteolytic and osteoblastic osteogenic sarcoma. In its age incidence, main sites of origin, behaviour, its radiological and macroscopic appearances it appears to be characteristic, although in a broader sense it may be regarded as a variant of the osteogenic sarcoma group. For this reason the cases of chondrosarcoma in the Bristol Bone Tumour Register have not been included in the series of tumours here examined.

On perusal of the literature on osteogenic sarcoma it appears that where other attempts at histological grading have been made, e.g., Simmons (1939), here again this has mainly been done on considerations of cell type and stromal characters. It is probable that these writers have been influenced by the approach of histologists to the group of fibro-sarcomata, where again the main histological grading has been based almost entirely upon cell morphology and the amount of stromal material produced by the neoplastic cells. This would seem to be the case in the grading of sarcomata adopted in such papers as those of Quick and Cutler (1927), Grynfeltt (1929), Geschickter (1932), and Broders, Hargrave and Meyerding (1939) to mention a representative few.

The present position may be more clearly shown by quoting from a paper by Coley and Pool (1940): " Microscopically, the grade of malignancy is demonstrable by a uniformity of pleomorphism of the cell type, and we feel that in a given case, the most highly malignant area seen under the microscope should determine the grade". The latter point is undoubtedly true, but it is suggested that the fundamental factor which dominates the selection of the " most highly malignant area" is only appreciated in a general way, i.e., the degree of mitotic activity of the neoplastic cells. It is a pathological truism to state that the presence of numerous mitotic nuclei in a section with concomitant atypism is indicative of a high degree of malignancy. The main purpose of this present paper is to define this well-appreciated fact in more precise terms and to focus attention more clearly upon its significance. Coley and Pool (1940) rightly draw attention to the other factors of a gross pathological nature which should influence the decision as to the grading of any particular sarcoma. The inspection of macroscopic pathology must largely be consequent upon either open operation, amputation or autopsy, and such details are not likely to be available to the histologist at the time of diagnosis. Some of these features can, it is true, be visualised by X-ray plates. However, the interpretation of these is mainly the proper province of the radiologist, and they are seldom to be seen in the routine pathological laboratory. For the fortunate histo-pathologist who may be granted access to such aids, the position is only improved to such an extent as the experience which he may have in the interpretation of skiagrams, which is a field with

many pitfalls. The ideal state no doubt is the close liaison between surgeon, radiodiagnostician, radiotherapist and histologist with personal conference in each and every case. Such states of collaboration seldom exist under conditions of normal routine work.

The histological specimens from the present series of cases have been considered under the following headings:

1. Predominant cell type and its morphological characteristics; the type and amount of concomitant stroma.
2. The degree of nuclear mitotic activity present in selected areas of each sarcoma.

MATERIALS AND METHODS.

The cases comprise those which have been referred to the Bone Tumour Register during the 5-year-period 1946 to 1951. To anyone working in this field the fallacies of diagnosis are only too well known, and the cases included have all been subjected to free discussion and unanimous acceptance as osteogenic sarcoma by the group of radiologists, radiotherapists, surgeons and pathologists which forms the working panel of the Register. In each case the diagnosis has been based upon consideration of the full history, clinical examination, adequate skiagrams and histological sections. In some instances the histological material is derived from biopsy, in others from surgical or autopsy specimens. This has been indicated in Table I, also the time relationship of the histological specimen to any pre-operative X-radiation.

For histological purposes the specimens were fixed, cut and stained by the usual routine methods. The evaluation of the degree of mitotic activity for each tumour has been based upon the calculation of the Mitotic Ratio (M.R.). This is given as the ratio of resting nuclei to one cell nucleus in active mitosis, and for the purposes of this investigation a nucleus was considered to be in mitosis from the time of rupture of the nuclear membrane until the reformation in the cell of two or more complete daughter nuclei. Owing to the presence in some sections of many multinucleated tumour giant cells, and the indistinct outlines of a large proportion of the polyhedral cells, the nucleus was taken as the unit rather than the cell. In each case a minimum of 2500 neoplastic cell nuclei were examined in each section, using a ruled internal screen in one ocular of a binocular microscope. The fine ruling of this screen subdivided the central area of the observed field into 25 equal squares, which enabled accurate counting to be performed at a magnification of 500. In each case the examination was confined to the most cellular areas in the sections available of each tumour, these being selected by careful scanning with the lower power objectives.

Repeat examinations were made of a number of histological specimens with a view to determining the degree of reliability of the method used to evaluate the mitotic ratio of each tumour. Typical results of these counts are shown in Table II. In each instance at least 2500 nuclei were examined.

General Features of Group of 36 Osteogenic Sarcomata.

Owing to the small number of cases available for this investigation, a brief analysis is given for comparison with the data obtained from much larger series of cases. Details are given in Tables I and II.

TABLE I.

No.	Case.	Biopsy, surgical, autopsy.	X-ray given before hist. specimen (tumour dose in röntgens).	Time interval between X-ray and specimen (days).	Further X-ray treatment given (tumour dose in röntgens, showing separate courses).	Remarks.
6	K. S—	Bi.	*nil*	..	308	Amputation, pre-operative.
10	P. C—	„	„	..	2000 to arm	No surgical treatment.
					2142 „	
					1215 „	
26	N. G—	„	„	..	6700 „	No surgery.
35	N. J—	„	250	1	4308 to leg	Amputation.
39	S. H—	„	*nil*	..	*nil*	„
43	D. C—	„	„	..	„	„
61	J. S. C—	„	720	1	3230 to leg	Local excision only.
					1980 „	
66	R. D—	„	3700	7	2830 „	No surgery.
					1140 to chest	„
70	A. L—	„	*nil*	..	4160 to pelvis	„
81	R. K—	„	428	2	5000 to arm	„
83	G. J—	„	*nil*	..	3400 to face	„
94	E. M. J—	„	„	..	2700 to leg	„
96	F. C. T—	„	450	2	3000 „	Amputation delayed 22 months.
					3000 „	
					2100 „	
					2100 to chest	
					1960 „	
					1820 „	
102	F. A. H—	„	*nil*	..	3220 to leg	No surgery.
					2320 „	
					1500 „	
106	J. W—	Aut.	„	..	*nil*	Local palliative surgery only.
136	Y. M—	Surg.	„	..	1500 to chest	Amputation.
					2000 „	Alive 3 years with pulm. metastases.

TABLE I (*Continued*).

No.	Case.	Biopsy, surgical, autopsy.	X-ray given before hist. specimen (tumour dose in röntgens).	Time interval between X-ray and specimen (days).	Further X-ray treatment given (tumour dose in röntgens, showing separate courses).	Remarks.
143	J. H—	Bi.	„	..	3400 to spine 900 „	No surgery.
145	K. C. W—	Surg.	„	..	1687 to chest 2460 to forearm	Amputation.
155	F. A. G—	Bi.	„	..	3120 to pelvis 3120 „	No surgery.
*157	D. J. A—	Surg.	8071	185	*nil*	Amputation, delayed 12 months.
163	D. R—	„	*nil*	..	„	„
169	M. S. A—	Bi.	„	..	3200, 2250 to lower abdomen	No surgery.
191	J. E. S—	Surg.	„	..	*nil*	Local excision only.
198	W. G—	„	„	..	„	Amputation.
203	S. C—	Bi.	„	..	8449 to arm	Diaphysectomy after 19 months.
212	R. M—	„	„	..	3865 to skull	No surgery.
213	E. C—	Surg.	„	..	*nil*	Amputation.
221	M. C. C—	Bi.	900	1	1900 to leg	„
226	A. R. O—	Aut.	*nil*	..	*nil*	No surgery.
230	C. H—	„	„	..	„	Path. fracture; no surgery.
250	M. G—	Bi.	450	5	4200 to leg stump 2500 „ 2000 „	Amputation.
273	T. H—	Surg.	1917	2	*nil*	„
299	F. H. B—	„	*nil*	..	„	„
317	C. P—	Bi.	„	..	2736 to forearm	No surgery.
337	J. A. B—	„	„	..	*nil*	Amputation.
338	A. D—	„	„	..	5744 to arm	No surgery.

* *Note.*—No. 157, D. J. A—: The specimen of tissue used for the histological section was an outlying recurrent nodule that had not been irradiated. A section of tissue derived from the irradiated area showed similar general morphological and structural features, with a mitotic ratio of 118 : 1 and a mean number of 300 cells per h.p. field. The figure from the more actively growing tissue has been used throughout the analysis, although the higher mitotic ratio would better fit the clinical grading.

TABLE II.

No.	Case.	Cell nuclei per h.p. field.	Mitotic ratio.	
66	R. D—	188	43	
		148	39	
		156	36	S.D. 2·408.
		124	41	
		112	39	Coefficient of variation
		144	40	6·068 per cent.
	Mean of 6	145	39·67	
157 (section of irradiated tissue)	D. J. A—	344	111	
		332	111	
		284	103	
		280	122	S.D. 9·411.
		296	118	
		269	134	Coefficient of variation
		273	124	7·954 per cent.
		321	123	
	Mean of 8	300	118·25	

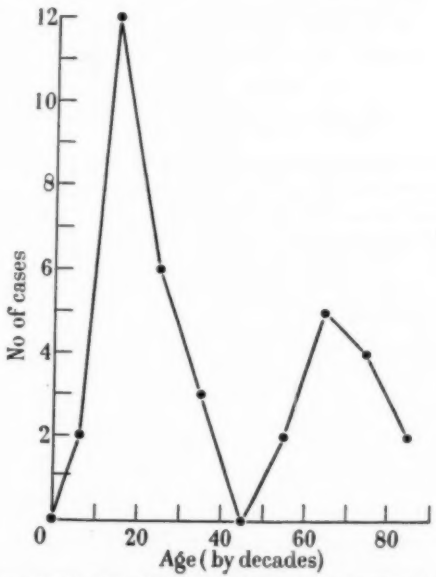

FIG. 1.—Age distribution of all cases of osteogenic sarcoma in this series.

1. *Age distribution.*—This is shown graphically in Fig. 1. The typical two-peaked curve shows the main incidence to be (*a*) in the second decade, 12 (33·3 per cent), (*b*) in the seventh decade, 5 (13·8 per cent).

2. *Sex distribution.*—Males, 25 (69·5 per cent), females. 11 (30·5 per cent).

3. *Site distribution.*—(i) Lower limb, 20 (56 per cent), of which 17 (47 per cent) were tumours, primary in the femur; (ii) upper limb, 8 (22 per cent); (iii) skull, 3 (8 per cent); (iv) axial skeleton, 5 (14 per cent).

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4. *Incidence of Paget's disease.*—This was present in 7 cases (19·5 per cent). Of these 7 cases the mean age was 70 years, with an average duration of 9·9 months.

5. *Five-year survival rate.*—Six cases lived for this minimal period. As there are also 8 cases included in the series that are still alive and when last reported were well, but for periods less than 5 years, the 5-year survival rate is best expressed as 6 in 28 cases—i.e., 21·4 per cent.

Two of these cases died at respectively 77 and 96 months with multiple pulmonary metastases, and the writer fully supports the view that 5 years is too short a period on which to base estimates of rate of cure in patients with osteogenic sarcoma.

These brief data may be compared with those published on larger series by the following: Christensen (1925), Coley and Pool (1940), the results of the New York Memorial Hospital series mentioned by Coley (1949), Geschickter and Copeland (1949), the series reported by the British Empire Cancer Campaign (1949), and Platt (1951).

As may be expected with a relatively small group of cases, there are some differences from the larger published series, e.g., an unduly high incidence of femoral tumours, but the broad agreement would suggest that this group may be regarded as a fair sample from which certain conclusions may tentatively be reached.

Clinical Grading of the Tumours.

The data on which the clinical assessment of degree of malignancy has been based has been considered from the following points of view:

1. Total time of duration of the morbid process. The data shown in Table III give the figures for each case as time in months. This has first been divided into two sections, ante- and post-diagnosis, and then the total combined time of survival to the date of the last recorded examination of the patient.
2. Site of origin of the growth.
3. Age of the patient at time of onset of symptoms.
4. Complicating factors, e.g., other skeletal disease.
5. A radiological subdivision of the tumours into those which are mainly osteolytic or osteoblastic. The histological analysis will be discussed in respect of these factors under the appropriate sections.

In attempting to arrive at some appreciation of the clinical degree of malignancy of each individual tumour for purposes of comparison, it was decided that the best clinical guide available could be obtained from consideration of the growth rate of each sarcoma, and also the total time of survival of each case from the date of presentation of the first relevant symptom. This method has certain inherent disadvantages, e.g., a tumour in an axial bone would prove more rapidly fatal than a similar one in the appendicular skeleton. It is also difficult to assess the influence of treatment upon the time of survival between diagnosis and death. Some indication of these factors is given later. In general terms the balance of prolonged life would be weighted heavily in favour of the patient with a tumour distally situated in a limb bone, rather than the patient with a sarcoma of the axial skeleton. However, in terms of total effect of the tumour upon the host, the total survival time is probably the best means of measurement of relative clinical malignancy in this type of growth.

Table III.

No.	Case.	Sex.	Age.	Site.	Duration of survival: 1. Ante-diag.	2. Post-diag. (months).	3. Total.	Alive or dead.	M.R.	Condition at time of death.	Other skeletal dis.	Type: poly. spin.
6	K. S—	M.	18	Femur	3	114+	117+	A.	450	—	..	P.
10	P. C—	M.	5	Humerus	1	7	8	D.	190	Sepsis; fungation of growth; haemorrhage	..	P.
26	N. G—	M.	13	Humerus, radius	10	65+	75+	A.	677	..	..	P.
35	N. J—	F.	14	Femur	2	80+	82+	A.	773	..	..	P.
39	S. H—	F.	19	,,	24	72	96	D.	888	Mult. pulm. mets.	..	P.
43	D. C—	M.	21	,,	2	96+	98+	A.	421	..	..	P.
61	J. S. C—	M.	15	,,	1	19	20	D.	206	Mult. pulm. mets. Active local tum.	..	P.
66	R. D—	M.	19	Tibia	1	10	11	D.	43	Mult. mets., lungs and liver	..	P.
70	A. L—	M.	58	Ilium	1	6	7	D.	180	Active local tum. in pelvis	P.	P.
81	R. K—	M.	22	Humerus	14	13	27	D.	195	No information	..	P.
83	G. J—	M.	73	Mandible	1	8	9	D.	65	Sepsis, fungation of growth	..	P.
94	E. M. J—	F.	73	Tibia	2	3	5	D.	48	Mult. pulm. mets.	P.	P.
96	F. C. T—	F.	38	Femur	13	35	48	D.	232	Local recce. sepsis. Mult. pulm. mets.	..	S.
102	F. A. H—	F.	70	,,	7	4	11	D.	132	Active tum. osseus and pulm. mets.	..	P.
106	J. W—	M.	27	Skull	3	1	4	D.	92	Active tum. sepsis; broncho-pneumonia	F.D.	P.
136	Y. M—	F.	24	Femur	1	76	77	D.	113	Mult. pulm. mets.	..	P.
143	J. H—	M.	9	Vertebra	2	13	15	D.	153	Active tum. compression of cord, paraplegia	..	P.
145	K. C. W—	M.	26	Femur	1	14	15	D.	108	Active tum. mult. pulm. and osseus mets.	..	P.
155	F. A. G—	M.	71	Ilium	3	8	11	D.	91	Mult. osseus mets.; pulm. collapse	P.	P.

TABLE III.—*continued.*

No.	Case.	Sex.	Age.	Site.	Duration of survival			Alive or dead.	M.R.	Condition at time of death.	Other skeletal dis.	Type poly. spin.
					1. Ante-diag.	2. Post-diag. (months).	3. Total.					
157	D. J. A—	M.	27	Femur	3	39+	42+	A.	73	..	..	S.
163	D. R—	M	13	,,	1	4	5	D.	88	Mult. pulm mets	..	S
169	M. S. A—	F.	62	Ilium	3	9	12	D.	198	Active tum. sepsis; mult. pulm. mets.	? P.	P.
191	J. E. S—	M.	38	Femur	3	28+	31+	A.	125	..	..	P.
198	W. G—	M.	73	,,	3	3	6	D.	174	Active tum.; delayed post-op. shock; haemorrhage	P.	P.
203	S. C—	F.	10	Humerus	8	28+	36+	A.	727	..	..	P.
212	R. M—	F.	62	Skull	2	5	7	D.	65	Active tum. sepsis. Paraplegia	P.	P.
213	E. C—	M.	16	Femur	1	24+	25+	A.	125	..	..	P.
221	M. C. C—	M.	14	,,	1	5	6	D.	135	Active tum.; mult. pulm and osseus mets.	..	P.
226	A. R. O—	F.	64	Vertebra	2	1	3	D.	168	Active tum.; compression of cord, paraplegia	..	P.
230	C. H—	M.	86	Femur	4	..	4	D.	91	Active tum. pneumonia (hypostatic); path. fract.	..	S.
250	M. G—	M.	14	,,	1	17	18	D.	83	Fungating local recce. sepsis	..	S.
273	T. H—	M.	15	Humerus	2	7	9	D.	46	Mult. pulm. mets.	..	P.
299	F. H. B—	M.	67	,,	2	9+	11+	A.	180	..	P.	P.
317	C. P—	M.	89	Radius	17	5+	22+	A.	926	..	P.	P.
337	J. A. B—	F.	36	Femur	13	4+	17+	A.	134	..	..	S.
338	A. D—	M.	60	Humerus	9	5+	14+	A.	183	..	..	S.

Notes.—Abbreviations: P. = Paget's disease. F.D. = Fibrous dysplasia.
P. = Polyhedral celled. S. = Spindle celled.

Mitotic ratio — expresses number of resting cell nuclei to one in mitosis.
Mean mitotic ratio of whole group, 238 : 1.

On reference to Table IV, which sets out the clinical grading of the 29 osteogenic sarcomata of polyhedral cell type, it will be noted—

1. That the cases graded I and II are juveniles with the exception of No. 317, C. P—, aged 89, and No. 191, J. E. S—, aged 38.

2. All these tumours (Grades I and II) have arisen in the appendicular skeleton.

3. The tumours of the axial skeleton have all been graded III.

In the group of spindle-celled sarcomata listed in Table V the age difference does not appear to apply at all, and there were no tumours of this type in the series sited in the axial skeleton.

Histological Grading.

The tumours have been graded I, II and III in ascending order of degree of malignancy, the annotation being largely based upon that adopted for tumours of epithelial origin. From considerations other than histological, e.g., site, concomitant skeletal disease, etc., it is possible also to indicate tumours which should also be regarded as Grade III plus. The various factors to be considered in the histological survey are compared with the clinical grading based upon the factors mentioned above.

Cell Type and Stroma.

It is possible to divide the whole group of 36 sarcomata into two major subgroups according to the main cell type present. These are :

A. Polyhedral and angular—29 cases.
B. Spindle-celled or fusiform—7 cases.

A. *Polyhedral-celled sarcomata.*

It is impossible here to escape the conclusion that the tumour-cell is primarily a neoplastic variant of the osteoblast. In a section of a well differentiated tumour the cells may show the somewhat basophilic cytoplasm and well-defined vesicular nucleus with 1 to 3 characteristic eosinophilic nucleoli seen in the normal osteoblast. The slight basophilia tends to diminish during mitotic activity, and the cell cytoplasm then stains a brighter pink with dyes of the eosin type. From observations on these tumours and other bony conditions, the writer has acquired the view that one of the essential functions of the osteoblast is the formation of osteoid tissue—the precursor of bone, from which under suitable conditions of substrate the formation of bone may occur, possibly with no further part being taken in the process by the osteoblast. Bone should be regarded as one of the end-products of a series of complicated and balanced chemical reactions, and modification of the several interacting factors may favour its deposition or dissolution. This view of the function of the osteoblast is largely in accord with that generally held, but modifies the function of the osteoblast as conceived by Leriche and Policard (1928), who mainly attributed to this cell a reactive role.

It has been noted also, however, that the osteoblast may be phagocytic as mentioned by Leriche and Policard (1928) quoting Dubreuil (1910), and by Bast (1924).

It is not uncommon to see active osteoblasts " dusted " with blood pigment derived from adjacent haemorrhage. This is not infrequently found in young

TABLE IV.—*Polyhedral-celled Osteogenic Sarcoma. Total of 29 cases* (A).

No.	Case.	Osteoid stroma.	Pleomorphism of cells.	Cells per h.p. field.	M.R.	Months total survival.	Clinical grading.	Remarks.
6	K S—	+++	+	..	450	117+	I	Small section.
26	N. G—	++	+	340	677	75+	I	Mod. new bone.
35	N. J—	+++	+++	132	773	82+	I	Very little new bone.
39	S. H—	+++	++	132	888	96	I	Much new bone.
43	D. C—	+++	+	168	421	98+	I	„ „
136	Y. M—	++	+	*432*	*113*	77	I (?)	Chondroid areas; see note *infra*.
203	S. C—	++	+	344	727	36+	I	Much new bone.
317	C. P—	+	+	264	926	22+	I	Small section.
	Means of Grade I (8)			228	619	75·4+ — 6 cases still living.		
61	J. S. C—	+++	++	240	206	20	II	Much new bone; cells big.
81	R. K—	+++	+	168	195	27	II	Chondroid; mod. bone.
143	J. H—	++	+	340	153	15	II	Little new bone.
145	K. C. W—	+	+++	244	108	15	II	„ „
191	J. E. S—	+	++	220	125	31+	II	Mod. new bone.
213	E. C—	++	+++	360	125	25+	II	„ „
	Means of Grade II (6)			260	152	22·2+ — 2 still living.		
10	P. C—	++	+++	268	120	8	III	No new bone; cells big.
66	R. D—	+++	+++	188	43	11	III	Mod. new bone.
76	A. L—	+	++	320	180	7	III	Mucinous stroma mainly.
83	G. J—	++	+	212	65	9	III	Very little new bone.
94	E. M. J—	+	++	300	48	5	III	No new bone in section.
102	F. A. H—	+	+	480	132	11	III	Little new bone.
106	J. W—	+	++	448	92	4	III	No new bone.
155	F. A. G—	+	+	232	91	11	III	„ „
169	M. S. A—	+	++	324	198	12	III	Little new bone.
198	W. G—	++	+	265	174	6	III	Mod. new bone.
212	R. M—	+−	+	196	65	7	III	No new bone.
221	M. C. C—	+++	+++	160	135	6	III	Much new bone.
226	A. R. O—	++	++	224	168	3	III	„ „
273	T. H—	++	+++	228	46	9	III	Little new bone.
299	F. H. B—	++	++	288	180	11+	III	No new bone.
	Means of Grade III (15)			276	120	8·0+ — 1 still living.		
	Means of whole group (29)			260	263	29·5		

Note.—BTR/136, Y. M—, has been placed in clinical Grade I in view of age (24), site of primary tumour (lower end of femur) and small size at time of operation—it was described by the pathologist as being "size of a bean." Although there was a long period of survival after amputation the mitotic ratio and eventual outcome of the case with the appearance of pulmonary metastases 40 months after her amputation suggest that clinical Grade II would perhaps be more appropriate.

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TABLE V.—*Spindle-celled Osteogenic Sarcoma. Total of 7 cases* (B).

There were no tumours in this group which warranted being clinically graded I.

No.	Case.	Stroma.	Pleomorphism of cells.	Cells per h.p. field.	M.R.	Months total survival.	Clinical grading.	Remarks.
96	F. C. T—	++	+	252	232	48	II	Collagen, some osteoid and little new bone.
157	D. J. A—	++	+−	356	73	42+	II	Collagen, osteoid and mod. new bone.
337	J. A. B—	+	+−	808	134	17+	II	Collagen only.
338	A. D—	+	++	344	183	14+	II	„ „
	Means of grade II (4)			440	158	30·5+ months, 3 still living.		
163	D. R—	+	+	400	88	5	III	Collagen mainly, little osteoid and new bone.
230	C. H—	++	+−	312	91	4	III	Collagen, much cartilage, little osteoid.
250	M. G—	+	+	284	83	18	III	Collagen only.
	Means of Grade III (3)			332	87	9·0 no survivors.		
	Means for whole Group (7)			392	128	20·9+		

callus and in Paget's disease. It is also generally accepted that the osteoblast, both normal and neoplastic, may produce phosphatase.

From the grounds that this cell therefore is actively concerned in the laying down of osteoid arises the essential stromal feature of this histological sub-type of osteogenic sarcoma, i.e., the characteristic osteoid stroma, which may also be accompanied by considerable amounts of collagen and ill-formed cartilage. However, the osteoid is the essential feature. This stromal material may, however, sometimes occur in considerable amount in a tumour which is predominantly spindle-celled, but is then usually less evident than the collagenous fibrous matrix produced by the spindle cells. Under suitable conditions the osteoid matrix may either calcify or ossify, more often the latter, and then produce the appearance of the osteoblastic or sclerotic type of tumour. It is thus possible to subdivide this group further into

i. Polyhedral-celled sarcoma—osteoblastic—22.
ii. Ditto —osteolytic — 7.

As may be expected, there is some overlapping in stromal composition between the polyhedral and spindle-celled groups. Of the seven tumours of the latter type in this series one could be classified as osteoblastic, the other six being osteolytic.

As the separation into blastic and lytic forms may largely depend on general radiological appearances, there is sometimes the difficulty of differentiating new tumour bone from new reactive bone and from calcified material. From the viewpoint of the histologist the essential histological structure of any neoplasm occurring in a bone is more characteristically shown in an area of active growth, the radiological manifestations of which are more frequently lytic than blastic. Hence this feature of the amount of bony matrix is not always readily appreciable from even a large biopsy. For this reason this subdivision will not be pursued further in the present analysis ; but it should be added, however, that a markedly bony stroma is often indicative of a tumour of relatively low malignancy, and this point should receive due consideration by the person who may attempt to correlate the histological, radiological and clinical findings in any particular case.

From inspection of Table IV the following points are evident :

1. The degree of cellularity of the tumour expressed in terms of the number of cells per standard field for the most cellular areas does not furnish any guide to the degree of malignancy.

2. Cellular pleomorphism is generally, but not invariably, an indication of a tumour of high-grade malignancy.

3. The converse relationship, whilst often observed, has numerous exceptions.

4. In an approximately quantitative assessment of the amount of osteoid stroma present in these sarcomata it is seen that there is usually a rich matrix in tumours of lower grade malignancy, and conversely a relatively smaller amount in the high-grade group. There are noteworthy exceptions, and the specimens showing perhaps this stromal feature in the greatest degree were those from BTR/66, R. D—, which were relatively acellular but had a well-developed matrix. Where this osteoid tissue is well developed it is usual for much of it eventually to ossify. There is some evidence suggesting that nuclear activity in a cell is accompanied by a lowered pH of the cytoplasm, possibly also producing some change also in the extracellular substrate in which the cells lie. Swenson (1946)

has shown that a low pH in a haematoma is associated with bone resorption in the region of a fracture. It appears therefore likely that the more acid tissue fluid which may exist in the region of rapid tumour growth is one of the factors which would influence the extent to which the osteoid stroma would be ossified. In support of this assertion it can be pointed out that it was a number of those tumours which showed a slow growth rate as evidenced by their mitotic ratio and clinical survival (Nos. 6, 26, 39, 43 and 203) that showed the most marked bone formation in their sections. There was also a moderate amount of bone present in the stromal tissues of Nos. 66, 213, 221 and 226, but generally this feature was less evident in the more actively growing tumours. It is emphasized that a change in pH of the substrate is only one of a number of factors concerned in this biochemical change. It is also wise to bear in mind a fallacy that may arise in sampling a bony tumour, as the more easily sectioned and hence less bony areas are frequently selected for routine diagnostic examination.

Quite a number of these sarcomata also show chondroid stromal areas which may either calcify or ossify, both changes often being seen in adjacent areas and largely governed by two factors :

i. Suitability of the substrate.

ii. Modification of the fine collagenous fibres of the chondroid material into a form which is denser and stranded, this change apparently being brought about by osteoblasts. In the absence of this latter change, the cartilage responds to its environment by calcification. In several tumours there were also patches of mucinous material, and all these stromal variants are indicative of the wide potential biochemical ability of the ancestral stem cell, the fibroblast. These features do not appear to have any special significance, from the viewpoint of grading.

5. The mean mitotic ratio of these sarcomata decreases as one passes from clinical Grade I to Grade III, This is much more evident in the separation of clinical Grade I from the tumours of Grades II and III. It may be noted here that based upon clinical data case BTR/136, Y. M—, has been graded I. It is not unlikely that this tumour should be re-graded II in view of the low mitotic ratio, and suggests that the long survival of this patient was a result of the prompt amputation which followed upon diagnosis. In this case the time period between the presenting symptom and radical operation was only one month, and the case may be regarded as a considerable surgical success.

B. *Spindle-celled group.*

These sarcomata, as compared with the polyhedral-celled group, are generally of a more uniform cellular structure. Tumour giant cells and multinucleated giant cells of distorted osteoclast type are much less frequently seen. Histogenetically they appear to be related to the fibroblast, which even in its neoplastic variant appears on occasion to be able to modify itself to become an osteoblast and form some irregular osteoid stroma. This feature was evident in 3 out of the 7 growths included in this sub-group, and in one of the tumours (BTR/157, D. J. A—) there was produced also a sufficient amount of new bone to warrant its classification as an osteoblastic osteogenic sarcoma.

The multipotent ability of the fibroblast which shows itself in the occasional appearance of some amount of osteoid, bony or chondroid stroma was recognised

by the American Registry of Bone Sarcoma in their 1939 (revised) classification, and they continued to regard intrinsic fibro-sarcoma as a variant of the osteogenic group (Ewing, 1939). Jaffe (1947), on the other hand, commenting upon this adheres to the stricter view and classifies fibro-sarcoma as a separate entity. From consideration of the small group of cases here reported it would seem that this differentiation is indistinct and serves no useful purpose.

Table V gives details of these 7 cases.

This group is far too small to be the basis for any definite conclusions, but the following tentative points emerge:

1. From considerations of cell morphology, stroma, and number of cells per unit area of the most cellular parts of the tumour in the sections examined, it is not possible to arrive at any useful histological grading.

2. On direct comparison by grades with the tumours in the polyhedral-celled group, those of the spindle-celled type show rather less stromal material, are on average somewhat more cellular but less pleomorphic, are mainly osteolytic, and the average time of survival of the patient afflicted with this type of bone sarcoma is rather longer in Grade II and about the same in Grade III.

3. The mean growth rates of these tumours graded clinically II and III in Table V as indicated by the group mean mitotic ratio agree fairly closely with those obtained for the comparable clinical grades of the polyhedral-celled group of sarcomata (Table IV).

4. In the spindle-celled group there were no tumours which appeared to merit a clinical or histological grading of I on comparison with those of the larger polyhedral-celled series. This appears to be confirmed on considering the figures obtained for the mitotic ratio in these growths.

5. On the overall picture obtained by comparing the tumours of clinical Grades II and III of each sub-group the following figures emerge:

			Duration.	Mitotic ratio, mean.	Cells/field, mean.
Group A.	Polyhedral-celled	(21)	12·1 months +	129 : 1	272
Group B.	Spindle-celled	(7)	20·9 months +	128 : 1	392

6. One may perhaps from this draw the conclusion that there is some evidence to suggest that in a series of these two histological types of osteogenic sarcoma if composed of tumours of average or more than average malignancy that the time of survival is rather longer in favour of the spindle-celled type. In this small series of cases, one obvious difficulty that has arisen has been the determination of mean survival time of the sub-groups, and it is fully appreciated that the figures shown are only minimal inasmuch as cases have been included which are still living. Where this has been done, it has been indicated by the presence of a plus sign after the figure denoting survival time in months. The inclusion of such cases was thought to be justifiable in view of the marked difference which can be shown in the prognosis in cases given a histological grading of I when the problem is approached purely from the histological angle. This is dealt with in the next section.

Analysis of Mitotic Activity of Whole Group of 36 *Sarcomata.*

It is widely known that a feature of the degree of malignancy of any tumour is the number of mitoses that can be observed in the nucleii of the component cells. It is also known that sarcomata, both spontaneous and experimental, are

mainly characterised by continued increase of tumour size, rather than by invasion of adjacent tissues. This continued growth is partly due to the multiplication of the number of cells of the tumour. For the purposes of this analysis it has been assumed that the mitotic ratio is an index of the growth rate of this series of sarcomata. Table III gives details of age, site, type of tumour, mitotic ratio and total duration of survival for the whole group.

From this table 9 cases can be selected in which the period of time between the appearance of the initial symptom and diagnosis is greater than 6 months. These are cases Nos. 26, 39, 81, 96, 102, 203, 317, 337 and 338, and all the patients had tumours of the appendicular skeleton. On reference to Table III again it will be seen that 7 of the 9 tumours showed a mitotic ratio considerably greater than the average for their appropriate clinical grade. Mean figures for this group of nine cases are given :

	Total months survival.	Months duration ante-diagnosis.	Mitotic ratio.
Mean of 9	40·9+	12·8	456

Conversely one may likewise select the type of case showing the other extreme, i.e., there were 11 cases in which the time period between onset of symptoms and diagnosis was one month or less. Mean figures for this group are given below. (Cases Nos. 10, 61, 66, 70, 83, 136, 145, 163, 213, 221, 250.)

	Total months survival.	Months duration ante-diagnosis.	Mitotic ratio.
Mean of 11	18·3+	Under 1	122

There is obviously some overlapping of individual figures between the two groups, and 2 tumours of the axial skeleton are included in the latter larger number.

Subject to individual differences in tolerance of patients and speed of diagnostic methods, one may assume that the more rapidly growing tumour will lead to the earlier symptomatology in tumours of a similar primary site. That being so, the difference in the degree of mitotic activity of the two groups of tumours is striking, as also is the difference in the mean time of survival of the former group as compared with the latter ; and whilst 5 of the former group are still living, there is only one survivor in the latter group. This degree of agreement between the mitotic ratio, ante-diagnostic period and total time of survival helps to render more evident the fallacy in the conclusion reached by Ferguson (1940), and supports the more reasonable explanation given by Coley (1949), that the more slowly growing tumour produces the less alarming symptoms. It would seem more rational to associate urgency of treatment with tumours of more rapid growth, i.e., Grades II and III, in which grades of malignancy the survival rates will be minimal, by whatever means the cases are at present treated. *Per contra*, in tumours of Grade I malignancy, occasional cures may be expected by radiotherapy only, e.g., No. 26, N. G—, and it is strongly suggested that the governing factor in the survival of any case of osteogenic sarcoma is the combination of site of origin and histological grading of the tumour.

Table VI shows the 36 cases of osteogenic sarcoma arranged in ascending order according to the mitotic ratio, i.e., in order of diminishing mitotic activity. This has been subdivided into three purely histological grades of malignancy,

into those cases with a mitotic ratio less than 100:1, from 100:1 to 400:1, and greater than 400:1. This may be taken as approximating to those of greater, average and lesser degrees of nuclear activity. Reference back to Tables IV and V will show that all the cases there clinically graded as III appear again as Grade III (histological) plus one case, BTR/157, D. J. A—, which was clinically graded II.

TABLE VI.—*Whole Group of 36 Osteogenic Sarcomata. Histological Grading.*

No.	Case.	Months total survival.	Mitotic ratio.	Cells per h.p. field.	Histological grade.	Clinical grade from Tables IV and V
66	R. D—	11	43	188	III	III
273	T. H—	9	46	228	III	III
94	E. M. J—	5	48	300	III	III
83	G. J—	9	65	212	III	III
212	R. M—	7	65	196	III	III
157	D. J. A—	42+	73	356	III	II*
250	M. G—	18	83	284	III	III
163	D. R—	5	88	400	III	III
155	F. A. G—	11	91	232	III	III
230	C. H—	4	91	312	III	III
106	J. W—	4	92	448	III	III
		(S.D. 10·9)				

* BTR/157, D. J. A— : Probably favourable result of treatment has led to unduly low clinical grading ; tumour in lower end of femur.

Group based on range of mitotic ratio of less than 100.
Mean survival period of grade III (11) — 11·4 + months. One still living (9·1 per cent.).

145	K. C. W—	15	108	244	II	II
136	Y. M—	77	113	432	II	I (?)
191	J. E. S—	31+	125	220	II	II
213	E. C—	25+	125	360	II	II
102	F. A. H—	11	132	480	II	III
337	J. A. B—	17+	134	808	II	II
221	M. C. C—	6	135	160	II	III
143	J. H—	15	153	340	II	II
226	A. R. O—	3	168	224	II	III
198	W. G—	6	174	265	II	III
299	F. H. B—	11+	180	288	II	III
70	A. L—	7	180	320	II	III
338	A. D—	14+	183	344	II	II
10	P. C—	8	190	268	II	III
81	R. K—	27	195	168	II	II
169	M. S. A—	12	198	324	II	III
61	J. S. C—	20	206	240	II	II
96	F. C. T—	48	232	252	II	II
		(S.D. 18·52)				

Group based on mitotic ratio range of 100 to 400.
Mean survival of Grade II (18) — 19·6 + months. Five still living (27·7 per cent.).

43	D. C—	98+	421	168	I	I
6	K. S—	117+	450	—	I	I
26	N. G—	75+	677	340	I	I
203	S. C—	36+	727	344	I	I
35	N. J—	82+	773	132	I	I
39	S. H—	96	888	132	I	I
317	C. P—	22+	926	264	I	I
		(S.D. 34·48)				

Group based on range of mitotic ratio greater than 400.
Mean survival of Grade I (7) — 75·1 + months. Six still living (85·6 per cent.).

The correlation coefficient for the whole series has been calculated for the two variates months of survival and mitotic ratio. The value obtained for r was 0·6156.

Histological Grade II in Table VI includes all those cases formerly given a clinical grading of II, plus one case BTR/136, Y. M—, formerly given a clinical grading of I and 8 others (Nos. 10, 70, 102, 169, 198, 221, 226, 299) which had been clinically graded III.

The 7 cases graded I (histological) in Table VI have in each instance been given a clinical grading of the same value.

It is evident then that on this basis any histological sub-division into Grades I and III is indistinct, and not always in accord with the clinical interpretation of such cases.

In the misfit in Grade III (histological) of Table VI (BTR/157, D. J. A—) the possible explanation can again be offered that the clinical grading based upon the consideration of survival time and other factors has been influenced by the treatment given to the patient.

Table VII shows the cases listed in Table VI as Grade II (histological) divided into those which appear as Grades II and III (clinical) in Tables IV and V. These two groups have been respectively designated Groups X and Y, and on comparison of these the following points can be seen:

Group X (Grade II histological, Grade III clinical).

1. The average age in this group is markedly higher than in Group Y, 51·6 years as compared with 28·9 years.

2. Excluding case BTR/299, F. H. B—, who is still alive and may eventually qualify for a more favourable clinical grading, the cases are with one exception all tumours of the axial skeleton, or of the upper end of the proximal long bone of a limb.

3. In the case of BTR/226, A. R. O—, the sarcoma at autopsy was of small size, but had produced collapse of a dorsal vertebra with marked angulation of the spine and compression of the cord. This may be compared with BTR/143, J. H—, in which a vertebral sarcoma occupied a more dorsi-lateral position and produced a very much larger soft tissue mass beneath the erector spinae muscles. These observations emphasize the bearing of the factor of site upon the clinical interpretation of any attempt to grade these tumours histologically.

4. Three cases of Group X (Nos. 70, 198 and 299) were complicated by well-marked Paget's disease, and there is some suspicion that sub-clinical Paget's disease may have been present also in case BTR/169, M. S. A—, in which the tumour had its origin in the ilium. No cases of Group Y showed any evidence of this skeletal complication.

5. After evaluation therefore of the mitotic ratio and allocation of the tumour to the appropriate histological grade, it is suggested that a proximal site, more particularly in the axial skeleton, advanced age or concomitant Paget's disease (these latter two features may possibly refer to the same aggravating feature), would indicate that the grading should be advanced to at least the next malignant grade, i.e., I to II, II to III and III to III+ when the case is considered *in toto*. *Per contra* it should be appreciated that a distal site, juvenility and a relatively low M.R. may be associated with a period of survival of 15 to 24 months. This is shown by case BTR/250, M. G—, aged 14, mitotic ratio 83 : 1, sarcoma of lower end of femur, who survived for a total period of 18 months. Another case supporting this view was BTR/157, D. J. A—, aged 27, mitotic ratio 73 : 1, sarcoma of lower end of femur, who was alive and symptom-free 42 months from

TABLE VII.—*Comparison of Cases in Table VI with* (a) *Dissimilar Clinical and Histological Grade;* (b) *Similar Clinical and Histological Grade.*

(*a*) Histological grading, II. Clinical grading, III. *Group X.*

No.	Case.	Bone.	Mititic ratio.	Age.	Type of tumour.	Months total survival.	Remarks.
102	F. A. H—	Femur	132	70	Poly.	11	Lower end of bone.
221	M. C. C—	,,	135	14	,,	6	Upper end of bone.
226	A. R. O—	Vertebra	168	64	,,	3	Body of vertebra.
198	W. G—	Femur	174	73	,,	6	Upper end of bone.
299	F. H. B—	Humerus	180	67	,,	11+	P. Lower end of bone.
70	A. L—	Ilium	180	58	,,	7	P. Axial skeleton.
10	P. C—	Humerus	190	10	,,	8	Upper end of bone.
169	M. S. A—	Ilium	198	62	,,	12	? P. Axial skeleton.
	Means of group (8)			51·6	..	8·0+	1 still living.

(*b*) Histological grading, II. Clinical grading, II. *Group Y.*

145	K. C. W—	Femur	108	26	Poly.	27	Lower end of bone.
191	J. E. S—	,,	125	38	,,	31+	,, ,,
213	E. C—	,,	125	16	,,	25+	,, ,,
337	J. A. B—	,,	134	36	Sp.	17+	,, ,,
143	J. H—	Vertebra	153	♀	Poly.	15	Lamina and transverse process.
338	A. D—	Humerus	183	60	Sp.	14+	Lower end of bone.
81	R. K—	,,	195	22	Poly.	27	Upper end of bone.
96	F. C. T—	Femur	232	38	Sp.	48	Lower end of bone.
61	J. S. C—	,,	206	15	Poly.	20	,, ,,
	Means of group (9)			28·9	..	24·9+	4 still living.

Abbreviations: P. = Paget's disease. Poly. = Polyhedral. Sp. = Spindle celled.

the date of the first symptom. However, even with this small group of cases it is evident that it is unusual for a long period of survival to be associated with a mitotic ratio of the tumour falling within the maximum observed range of 43:1 to 92:1. This feature can be well appreciated from Fig. 2, which depicts the per cent survival by years of the cases in the three histological grades of Table VI.

Histological Grade I of Table VI.

It would appear from the tabular data that it is possible to distinguish histologically in a series of cases of osteogenic sarcoma those tumours which may be graded I (histological). The mean mitotic ratio for the whole group of 36 sarcomata was 238:1 cell nuclei. If one selects as a separate group (P) the 7 cases in which the mitotic ratio is markedly greater than the average the following figures are obtained (No. 6, 26, 35, 39, 43, 203, 317) :

Total.	Months survival.	5-year survival.	Mean M.R.
Mean of 7	75·1+	5 (71 per cent.)	695 : 1
Mean of 29	14·1+	1 (3·4 per cent.)	128 : 1

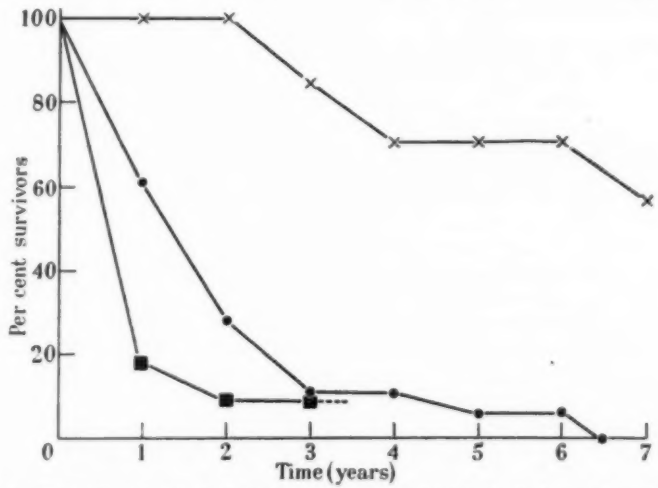

FIG. 2.—Percentage survival of Grades I, II and III (histological) shown at yearly intervals.

×——× Histological Grade I (7). Mean survival 75·1 months.
●——● Histological Grade II (18). Mean survival 15·8 months.
■——■ Histological Grade III (11). Mean survival 11·4 months.

The marked difference in the prognosis in these separated groups of cases can again be well seen from Fig. 2. It would be of the greatest value to subject many further cases to this type of analysis, to confirm if possible the significance of the mitotic ratio and to find its mean value based upon the examination of a much larger series of tumours. The number of sarcomata found in this present series of 36 tumours in which the mitotic ratio is appreciably greater than the mean figure is 19·4 per cent of the total. It will be noted that this figure is approximately of the same order as the 5-year survival rate (21·4 per cent). Reference

to Table I shows the treatment which was given to these 7 cases. As can be seen, surgical success may occur in the treatment of cases of higher-grade malignancy (II and III), but such is unusual. The analysis presented above suggests that calculation of the mitotic ratio at the time of diagnosis by the pathologist will enable him to indicate the grade of his histological malignancy, and hence some idea of the prognosis may be determinable for any particular case with more certainty. If this method can be firmly established it should be of great assistance in the difficult decision over upper limb amputations. There are 3 such cases in this series (No. 26, 203 and 317), 2 of which have been treated by radiotherapy only, and one by radiotherapy followed after 18 months by diaphysectomy on account of renewed and progressive pain. In cases No. 26 and 203 a good prognosis can be given with reasonable confidence; this, however, is rather more guarded in case No. 317 in view of the patient's advanced age and concomitant Paget's disease, although in this latter case the site in the lower end of the radius of the tumour does not suggest a rapidly fatal issue.

In view of the figures given for the mitotic ratio in this group of sarcomata, it is interesting to compare these with figures obtained for similar investigations on non-neoplastic specimens. (Table VIII).

TABLE VIII.—*Mitotic Ratios in Non-neoplastic Specimens.*

No.	Initials.	Condition.	M.R. osteoblasts.	M.R. fibroblasts.
343	M. G. C—	Callus	>5000 : 1	>5000 : 1
370	H. J. O—	Myositis ossificans	>2500 : 1	>2500 : 1
371	G. F. M—	Osteochondroma	3300 : 1	>2500 : 1
341	O. J. N—	Osteoma	1250 : 1	>2500 : 1
352	M. E—	Fibrous dysplasia	>2500 : 1	>2500 : 1
339	B. A—	„ „	>2500 : 1	>2500 : 1

In each of these specimens of tissue there was marked cellular functional activity with the formation of much new osteoid and bone. The mitotic ratio in all these specimens is of a different order from those found in the general run of the group of osteogenic sarcomata. Apart from the histological features, appreciation of this fact may be of considerable assistance when attempting to reach a decision on an atypical section of bony material. That the very slightly greater degree of mitotic activity was found here related to the osteoblast, and not the fibroblast, if confirmable, will strengthen the concept of the histogenesis of the polyhedral-celled type of osteogenic sarcoma, and support the assertion of Geschickter and Copeland (1949) that such tumours are derived from osteoblasts.

CONCLUSIONS.

1. The series of cases of osteogenic sarcoma here reported, although small in number, appears to conform with the main features of much larger groups formerly published.

2. By the application of histological methods only, it is possible to grade these sarcomata I, II and III in ascending order of malignancy, the scheme adopted being applicable to tumours of either predominantly a polyhedral or spindle-celled sub-type.

3. The amount and type of tumour matrix, whilst of some indicative value, are not reliable criteria for assessing the histological grade of these growths.

4. The degree of cellularity and morphology of the component cells of the tumours are features of some value to be considered in grading osteogenic sarcomata, but the main factor to be observed is the degree of nuclear activity which may be estimated by the mitotic ratio.

5. The mean mitotic ratio for this series is 238 resting nuclei to one nucleus in mitosis. Analysis of the group gives the following ranges :

Grade I : M.R. greater than 400 to 1.
Grade II : M.R. from 400 to 1 down to 100 to 1.
Grade III : M.R. less than 100 to 1.

6. It appears unusual to find a short total survival period of any case associated with a M.R. greater than 400 to 1. The converse may be encountered, and is probably an index of other features of the tumour, e.g., site, patient's age, etc., or of the effects of treatment.

7. It is suggested that the site of the tumour and its histological grading are the factors bearing most significantly upon the prognosis, and it has been found that the five-year survival rate is almost identical with the incidence of tumours of histological Grade I.

8. The mean mitotic ratio for this group of sarcomata is markedly different from that which has been found in the examination of presumably related cells in non-neoplastic conditions.

The author wishes to record his great indebtedness to the late Dr. S. Bryan Adams, sometime Director of the Radiotherapy Department of the Bristol Royal Hospital, through whose energy and initiative the Bristol Bone Tumour Register came into being. He was also directly and personally concerned in the treatment of many of the earlier cases, and the collection of their clinical and radiological records. Much thanks are also justly due to the members of the Committee of the Register for their co-operation in the collection and discussion of all the cases included in this series. Material has been derived from the source-indicated below, and to the surgeons, radiotherapists, radiologists and pathologists who have referred cases to the Register, due recognition and thanks for their assistance are here rendered :

1. Radiotherapy Department and Department of Surgery, Bristol Royal Hospital. Cases No. 6, 10, 26, 35, 39, 61, 66, 70, 81, 83, 94, 96, 102, 145, 155, 157, 169, 203, 212, 213, 273, 317, 338.

2. A. L. Eyre-Brook, Esq., F.R.C.S. Cases No. 43, 163, 198, 230.

3. Dr. R. C. Hadden. Radiotherapy Department, Royal Devon and Exeter Hospital. Cases No. 136, 250.

4. J. Bastow, Esq., F.R.C.S. Royal United Hospital, Bath. Cases No. 143, 221, 299.

5. J. F. H. Stallman, Esq., F.R.C.S. Gloucestershire Royal Hospital. Case No. 191.

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SPONTANEOUS MAMMARY ADENOMA AND CARCINOMA IN RIIIb MICE DEPRIVED OF MILK AGENT.

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THE suggestion was previously made that nodular adenomatous hyperplasia in mammary glands of mice of the RIII (Paris) strain, and possibly others, might provide a more sensitive and an earlier indication of the action of Bittner's milk agent than do actual tumours. Evidence obtained under experimental conditions of forced activation of the glandular response to combined influence of hormone and agent was given in support of this suggestion. It seemed probable also that spontaneously occurring nodules as well as those forcibly stimulated would afford reliable evidence of the action of the agent. The validity of such evidence would depend upon complete absence of spontaneous adenomatous nodules in the same strain deprived of agent. In sublines of the RIIIX strain (renamed RIIIb in accordance with international usage), which had been freed from agent by cross-suckling, no nodules had been found in the mammae of young unmated females forcibly stimulated, but two hyperplastic nodules were seen in 2 out of 44 females of the old breeding stock (Pullinger, 1947). Doubt thus arose whether the cross-suckled sublines were in fact entirely agent-free or whether, in old females, these nodules had some other cause. A survey of the mammae of all old females was in progress and was continued and combined with experiment in an endeavour to find an answer. The same problem, the stimulus for hyperplastic nodules in the Zb strain (C3H cross-suckled to deprive it of milk influence), was investigated by Huseby and Bittner (1946), who came to the conclusion that all three factors, namely the milk tumour influence, ovarian hormone and susceptibiltiy that are responsible for the development of heritable mammary tumours in mice are also necessary for the development of these alveolar hyperplasias. The same authors gave an account of the morphology of nodules found in the mammae of mice in former high cancer strains and their hybrids after deliberate exclusion of the agent by cross-suckling. The strains examined were C3H and A, designated Zb and AX. They compared the nodules with those in high cancer strains described by previous authors before knowledge of the milk agent existed. In the course of the present survey of old RIIIb (formerly RIIIX) breeders similar types of nodule were seen with the exception of those described as inflammatory. Focal acinar proliferations were often but not invariably discretely lobular. The squamous-celled nodules found in old RIIIb females have been described and their origins discussed (Pullinger, 1949). In relation to the problem of the presence and action of the milk agent these squamous-celled proliferations are not relevant, for it is generally agreed that tumours due to Bittner's agent are characteristically acinar formations and that

squamous-celled carcinomas are rarely associated with the agent (Dunn, 1945; Bonser, 1945; Kirschbaum, 1949). Some squamous-celled carcinomas reported by others in agent-free strains have since been tested for agent, and in no example has it yet been found either among spontaneous growths (Heston, Deringer, Dunn and Levillian, 1950) or in any that have been induced (Dmochowski and Orr, 1949; Bittner and Kirschbaum, 1950).

The present contribution includes an investigation into the incidence of adenomatous nodules in various groups of females of both the original RIII and the cross-suckled RIIIb strains and into the cause of those in the latter (Table IV). Multiple nodules were found in the mammae of all unmated RIII females by 9 months old; none was found either in young unmated or breeding females of the RIIIb sublines, but solitary or a few adenomatous nodules were detected in 19·3 per cent of RIIIb breeders from 12 to 25 months old. Experiments were done to determine whether these adenomatous nodules in old breeders were dependent for their maintenance on circulating ovarian hormone, or on the milk agent, despite its presumed absence. The nodules may be precursors and very early stages of tumours morphologically indistinguishable from those associated with the agent, hence the value of deciding what factors cause them. Reference is also made to the mixed squamous and adenomatous nodules found in the strain and to other spontaneous tumours.

A continuing hormonal influence having a focal effect in old involuted mammae was sought for by ovariectomy. Adenomatous nodules persisted nevertheless for at least 4 months, thus indicating their independence of circulating ovarian hormone once they have been formed (Table I). The impulse for their origin remains undecided.

The presumption that the milk agent was absent and therefore could not account for the adenomatous nodules was based on the derivation of the mice from sublines resulting from brother and sister, and occasionally parent and offspring, matings of progeny of one male and one female of an RIII litter which had been cross-suckled in 1944. Although adenomatous nodules had been found, no mammary tumours had yet arisen when these experiments were begun. Subsequently two adenocarcinomas arose in 2 out of 472 females that lived for 10 months or more, one at 13, the other at 17 months of age. These two tumours were tested for agent. No evidence of its presence was found in susceptible test mice (Tables II and III). No cause for them or for the adenomatous nodules was found.

No provision could be made for observations on any heritable factor that might be associated with the nodules or tumours because the stock served as source for young mice for other experiments. Evidence of parent and progeny relationships in mammary tumour incidence in agent-free strains has been discussed by Bittner (1942), by Heston (1945) and by Dmochowski (1951).

METHODS.

Besides the pure line RIIIb strain a few sublines were developed from crosses within the strain. This was done at one time in an effort to improve breeding qualities. Almost at once the original pure line mice began to give a greater yield of progeny, so the sublines derived from crosses within the strain were discontinued. Old breeding females of all sublines were included in the survey, extending from 1944 to 1951.

Breeding was usually limited to 5 to 7 litters in order to ensure survival of the mice to old age. About 5 per cent were sterile. Many were by chance force-bred up to a maximum of 8 litters owing to their habit of destroying their young. The actual average number of litters was 4 to 5. Breeders were then segregated to allow the mammae to involute, thereby permitting detection of nodular hyperplasia of various sorts. Under these conditions involution is completed in this strain, leaving main and a few branch ducts with tapered ends and no acinar structures. The ducts are closed and devoid of secretion.

Test of influence of ovarian hormone on adenomatous nodules.

As females reached 20 months of age they were divided in successive batches between three groups kept roughly equal in numbers as the experiment progressed. Group I was killed at 20 months to ascertain nodule incidence in intact mice of this age; Group II was ovariectomised at 20 months and killed at 24 to see whether adenomatous nodules expected on the basis of observation of the first group would survive in the absence of circulating oestrogen. Several of this group died immediately or within 24 hours of operation. These were added to Group I. Group III mice were killed at 24 months to find the incidence at this age, and thus determine by comparison with mice of Group I if any reduction or increase in nodules could be expected between 20 and 24 months (Table I).

TABLE I.—*Lack of Influence of Ovarian Hormone on Persistence of Adenomatous Nodules in Old RIIIb Parous Females.*

	Total number of mice.	Number of mice with acinar nodules.	Total acinar nodules.
Group I:			
Killed at 20 months	52	14	22
Group II:			
Ovariectomy at 20 months. Killed at 24	50	13	17
Group III:			
Intact. Killed at 24 months	50	18	23

Ovariectomy was done under bromethol anaesthesia. When mice were killed remnants of uteri and vaginae were examined to ascertain that they had atrophied. Cornification tests to exclude the possibility that oestrogen was being produced in suprarenals subsequent to ovariectomy were not done because they had been found to be unnecessary in adult mice in this strain on previous occasions. All 10 nipple areas were examined as previously described (Pullinger, 1947). Those containing hyperplastic nodules of any kind were mounted and kept. Owing to the presence of mixed squamous and adenomatous nodules particular care was taken on occasion to prove the purely acinus-forming nature of the adenomatous nodules. This was done by examining serial sections through whole nodules after excluding the presence of squamous acini by polarised light. Nine acinar nodules were thus examined from intact mice and 2 from mice deprived of their ovaries 4 months previously. It was not possible to subject all predominantly acinar new formations to serial section. Therefore they were judged to be purely or mainly acinar on the basis of the polarising test used

before (Pullinger, 1949), and from experience of a total of 60 nodules derived from various surveys in hand and cut in serial section by the author.

Results are recorded in Table I, from which it will be seen that the mammae of 14 out of 52 intact breeders of 20 months old contained acinar nodules ; thirteen out of 50 ovariectomised old breeders and 18 out of 50 intact breeders killed at 24 months contained adenomatous nodules. Two ovariectomised mice, not included in the Table because they had to be killed at 22½ and 23 months, developed mammary tumours, the one a pure squamous-celled carcinoma, the other a mixed squamous and adenocarcinoma (adeno-acanthoma). Adenomatous nodules persisted and mammary carcinomas arose and progressed in spite of ovariectomy.

Biological test of two adenocarcinomas for Bittner's milk agent.

Grafts of the 2 adenocarcinomas, Tvi.21 and Yx.30, were tested as soon as susceptible young became available. Tvi.21, a rapidly growing alveolar type of adenocarcinoma, arose at the tip of the 4th left nipple region at 17 months of age. First grafts grew in 12 out of 12 RIIIb males. Cells of this tumour have been examined by ultraviolet microscopy by Ludford and Smiles (1950). Grafts have never regressed. The tumour is now in its 38th generation. Yx.30, also an alveolar adenocarcinoma, arose in the left 2nd or 3rd nipple region at 13 months of age. First grafts grew, but much more slowly than the other tumour, in 8 out of 8 RIIIb males.

Tumour extracts equivalent to 0·05 g. of first, second and fourth generation grafts of Tvi.21 were injected intraperitoneally into 16 RIIIb females and 21 C_{57} × RIIIb F.i. hybrids of less than 1 month old. Extracts were made by grinding the tumours with sterile sand and distilled water, spinning at 2000 r.p.m. in a Hearson centrifuge for 10 minutes, removing the supernatant fluid and spinning this in an Ecco centrifuge at approximately 8000 g. for 15 minutes. The final supernatant fluid was warmed to 37° C., and 0·5 ml. was injected intraperitoneally into each of the young mice.

Tumour extracts similarly prepared and equivalent to 0·05 g. of 1st generation grafts of Yx.30 were injected intraperitoneally into 5 RIIIb and into 12 C_{57} × RIIIb F.i. hybrid females. Extracts equivalent to 0·025 g. of 1st generation grafts were injected intraperitoneally into 4 RIIIb and 11 C_{57} × RIIIb F.i. females. The smaller quantity was due to limited tumour material owing to slow growth of grafts. Equal quantities of RIII spontaneous tumour extracts were injected into litter mate controls in both experiments in order to prove susceptibility of the test mice. All injected females were then force-bred with agent-free litter mates in boxes of not more than 6 per box. Litter records were kept.

Results in terms of tumour incidence are recorded in Tables II and III. No tumours arose in any of the mice injected with extracts of Tvi.21 or Yx.30, whereas the majority of survivors up to 10 months of their litter mates injected with extracts of spontaneous RIII tumours had been killed while bearing tumours by 15 months after injection. All surviving mice were killed at the end of 24 months of experiment. The mammae of as many as possible of the RIIIb mice injected with test tumours were examined in bulk-stained preparations. It will be seen from Tables II and III that the F.i. hybrids injected with RIII tumour extracts were more satisfactory test animals than were the pure line

RIIIb females in respect of tumour development. This greater responsiveness of hybrids to the action of agent may be related to their greater fertility, as

TABLE II.—*Summary of Biological Test for Agent in Spontaneous RIIIb Tumour Number Tvi.21.*

Test mice.	Months of experiment.	Control of susceptibility with RIII tumour extracts. Number of mice used, 22		Extracts of tumour grafts of Tvi.21 undergoing test. Number of mice used, 21	
		Number of mice alive.	Number dead with tumours.	Number of mice alive.	Number dead with tumours.
C_{57} × RIIIb F1.	10	13	7	20	0
	15	0	20	20	0
	19	..	..	18	0
	24	..	..	12	0
		Average number of litters, 11.		Average number of litters, 15.	
	Months of experiment.	Number of mice used, 15.		Number of mice used, 16.	
		Number of mice alive.	Number dead with tumours.	Number of mice alive.	Number dead with tumours.
RIIIb	10	11	3	14	0
	15	6	8	14	0
	19	3	10	14	0
	24	0	10	0	0
		Average number of litters, 3.		Average number of litters, 5.	

TABLE III.—*Summary of Biological Test for Agent in Spontaneous RIIIb Tumour Number Yx.30.*

Test mice.	Months of experiment.	Control of susceptibility with RIII tumour extracts. Number of mice used, 22		Extracts of tumour grafts of Yx.30 undergoing test. Number of mice used, 23	
		Number of mice alive.	Number dead with tumours.	Number of mice alive.	Number dead with tumours.
C_{57} × RIIIb F1.	10	7	15	19	0
	15	0	22	15	0
	19	..	..	5	0
	24	..	..	2	0
		Average number of litters, 9–11.		Average number of litters, 18	
	Months of experiment.	Number of mice used, 8.		Number of mice used, 9.	
		Number of mice alive.	Number dead with tumours.	Number of mice alive.	Number dead with tumours.
RIIIb	10	5	2	6	0
	15	0	3	4	0
	19	..	..	4	0
	24	..	..	1	0
		Average number of litters, 3–4		Average number of litters, 5–6	

shown in the tables by the average number of litters. The hybrids bore about 3 times as many litters as the RIIIb mice, 3 of which were sterile in the two experiments. According to von Mühlbock (1950) force-bred females of the

dilute brown strain and their hybrids which bore more than 3 litters had a higher incidence of mammary tumours than those with less. The parous RIIIb females attained this average with 4 to 5 litters. Of mice injected with RIII tumour extracts 13 out of 23 developed mammary tumours. The mammary glands of all 7 which were examined, out of the remaining 10, contained multiple adenomatous nodules, one as many as 30. Thus nodule incidence was increased in the RIIIb test mice by injection with RIII tumour extracts.

Nodule incidence was not increased in the RIIIb mice which had been inoculated with extracts of the 2 tumours undergoing test for the agent. Of 20 mice in the two experiments which were available for examination, the mammae of 3 contained acinar nodules (15 per cent), 8 contained nodules of other types, and 11 (55 per cent) were free of all nodules. These figures are near the normal incidence. Nodules in hybrids are not recorded because there was no basis for comparison.

Thus neither from tumour incidence nor from nodule incidence was there any evidence of the presence of Bittner's milk agent in the 2 test tumours.

The validity of results of tests in which grafts are used as suspected source of agent rather than primary tumours may be questioned. The majority of authors have been able to detect the milk agent in extracts of grafts derived from tumours which were known to have contained it in the first instance. Dmochowski (1949), who reviewed the various reports, has himself obtained evidence of the presence of agent after 42 serial transplantations. On that occasion he used multiple injections, but both he and others have succeeded with single injections of graft extracts. In a total of 69 test mice in the two experiments here recorded, not one tumour arose.

These results are in accord with those of other workers who have tested extracts of primary spontaneous mammary adenocarcinomas in other agent-free strains (Andervont and Dunn, 1950; Heston, Deringer, Dunn and Levillian, 1950; Dmochowski, 1951). No evidence of the presence of agent was found by any of the authors. Two tumours tested by Andervont and Dunn and 18 by Heston *et al.* (personal communication) were typical adenocarcinomas.

Assessment of the value of adenomatous nodules as indicators of the action of the milk agent.

A comparison of the spontaneous incidence of adenomatous nodules in various groups of RIII and RIIIb mice is given in Table IV. It will be seen that among virgin females of the RIII strain multiple nodules were present in 100 per cent at 9 months old. They were found less constantly below this age. The relative reduction in number of nodules at 13 months after ovariectomy at 9 months is probably due in part to the difficulty of making accurate counts when the effect of ovarian hormone is still to be seen.

Among young virgin females of the RIIIb strain deprived of milk agent no adenomatous or other mammary nodules were found. Among RIIIb breeders up to 11 months old none was seen, but they occurred in 19·3 per cent in the age-group 12 to 25 months. No morphological basis for distinguishing nodules from the two sources, agent-containing and agent-free mice, has been detected. On the whole, acini of nodules in the agent-free strain contain less secretion and are more often discretely lobular in arrangement, but these distinctions do not always hold true.

TABLE IV.—*Incidence of Spontaneous Adenomatous Nodules in Females of RIII and RIIIb Strains.*

	Mouse strain.					
	RIII.			RIIIb.		
Reproductive state :	Virgins spayed at 2 months.	Virgins not spayed.	Virgins spayed at 9 months.	Virgins not spayed.	Breeders.	
					Young.	Old.*
Age when killed	9 months	9 months	13 months	9 months	6–11 months	12–27 months
Number of mice	25	35	24	24	30	425
Mice with adenomas	6	35	24	0	0	82
Percentage with adenomas	24	100	100	0	0	19·3
Total adenomas	19	1001	538	0	0	88
Average number per mouse affected	3·1	28·6	22·4	0	0	1·0
Total adenocarcinomas	0	4	9	0	0	2

* Including some spayed.

The combined data make it clear that adenomatous nodules are a sure indication in this strain of the action of the milk agent in young females only. These may be parous or virgin less than one year old. In breeders over this age the nodules may have another cause. This other cause does not appear to be excessive ovarian stimulation.

Mixed adeno- and squamous carcinomas (adeno-acanthomas), nodules and other tumours.

Among 472 breeding females that lived for 10 months or longer, 6 mammary carcinomas (2 adeno-, 2 squamous-celled, 2 adeno-acanthomas) were found. There were 10 spontaneous sarcomas, and a small percentage of lymphomas and pulmonary adenomas. The types of mammary tumour, with age-incidence in brackets, are shown in Table V.

TABLE V.—*Types and Age Incidence of Mammary Tumours in 472 RIIIb Females.*

	Number of tumours.			
Type of tumour.	Macroscopic. (Age in brackets.)	Total.	Microscopic. (Age in brackets.)	Total.
Adenocarcinoma	1 (17), 1 (13)	2	1 (27)	1
Squamous carcinoma	1 (25), 1 (22)	2	1 (21), 1 (22) 1 (16), 1 (13)	4
Adeno-acanthoma	1 (23), 1 (14)	2	1 (24), 1 (12)	2

The microscopic tumours were something between nodules and frank tumours. They were just those structures which so strongly suggest a transition from benign to malignant. The percentage of mice with nodules of mixed type was about 26, and of mice free of nodules of any kind 61.

No tumours of any kind were found in males, but few males were kept as long as the females.

CONCLUSIONS.

The reliability of spontaneous adenomatous nodules as indicators of the action of the milk agent in the RIII strain depends upon the age of the mice.

When these nodules are present in mammae of young females of this strain of less than 12 months old, whether virgin or parous, they appear to provide reliable evidence of the action of the agent. This conclusion is more certain if oestrogen has been excluded by ovariectomy several months prior to examination of the mammae.

Breeders of the RIIIb strain deprived of agent, of 12 months of age or more, developed 1 to 3 adenomatous nodules in the 10 nipple regions. Since these nodules were not associated with agent in the tests recorded, it is clear that they do not invariably provide evidence of the presence of agent in old breeders. They may be due to some other unknown cause. Multiple adenomatous nodules in old breeders of this substrain would nevertheless provide presumptive evidence, requiring confirmation, of the presence of the agent.

Thus for purposes of detection of agent by inoculating suspected substances into susceptible test mice and using the adenomatous nodule as evidence of the action of agent, only young RIIIb virgins or breeders under 12 months old would be suitable as test animals. That is to say, they would have to be killed and examined at about 9 to 11 months old when positive results could be expected and before the nodules of unknown origin begin to appear. Only if counts of nodules were required would ovariectomy have to be done because the naturally occurring hormone under normal conditions does not cause a confusing degree of focal acinar proliferation. The saving of time, however, might not be very valuable because by 15 months of age the majority of breeders develop gross tumours (Tables II and III). For quantitative work the test might be of use. Forcible oestrogenic activation might reduce the time of nodule appearance due to injected agent, as it did when agent was present naturally (Pullinger, 1947), but this test requires ovariectomy.

Other strains than the RIII have not been sufficiently studied, so far as one knows, to allow the generalisation that multiple adenomatous nodules in young virgins or breeders of any strain are an expression of the action of the agent. Knowledge of the presence or absence of these nodules in young females is lacking. Such observations as are on record (Huseby and Bittner, 1946) support the generalisation as applied to the Zb strain (C_3H cross-suckled). The mammae of a large proportion of old breeders of most strains that have been freed of agent (except the AX examined by Huseby and Bittner) contain adenomatous and other nodules. In the C_3Hb cross-suckled strain adenomatous nodules (Jones 1951) and tumours (Heston, Deringer, Dunn and Levillian, 1950) are so numerous in old breeders that even multiplicity does not afford presumptive evidence of the presence of the agent.

The more varied morphology of adenomas and carcinomas in agent-free strains and their late age-incidence point to a cause or causes other than the milk agent.

It is clearly of importance to know the relationship, if any, of agent-free adenomas to carcinomas. Experiments to determine this relationship have been begun.

SUMMARY.

1. Nodules of purely adenomatous hyperplasia were found in about 19 per cent of parous females of 1 year old and over of the RIIIb strain deprived of Bittner's milk agent. Two adenocarcinomas occurred during the course of these observations among 472 breeders.

2. No evidence was found that the nodules or tumours in the mammae of old parous females of the cross-suckled RIIIb strain owed their persistence to ovarian hormone ; neither could their origin or persistence be attributed to the milk agent. No cause for them was found.

3. Adenomatous nodules, whether spontaneous or activated by ovarian hormone, provide valid evidence of the action of the milk agent only in young RIII females less than 12 months old, whether virgin or parous. In old breeders these nodules may be due to the agent or to other cause or causes unknown.

4. Spontaneous sarcomas developed in 2 per cent of all females of the RIIIb strain deprived of milk agent.

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A GLAND OF PREDILECTION FOR MAMMARY NODULES IN A STRAIN OF MICE DEPRIVED OF THE MILK AGENT.

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THE hyperplastic nodules here referred to comprise all varieties found in surveys of spontaneous nodules in previous publications (Pullinger, 1949, 1952). At the time these surveys were being made specimens of affected nipple regions were almost invariably mounted and kept. It gradually became obvious that one nipple region, the 2nd on either side, was more often the site of nodule formation than any other. When the surveys were complete, the 5 pairs of nipple regions were arranged together with the number of times they contained one or more hyperplastic nodules of the various sorts. Only those nodules are now included of which whole mounts were kept. The records were inadequate for grouping in 10 nipple regions as right or left sided. The period of time covered was continuous from 1945 to the end of 1950.

The number of mice with mounted nodule-containing nipple regions was 116; the number of the nipple regions 146. No record had been kept of 13 from which serial sections were made in the earlier stages of the survey; 16 more were incomplete or have been mislaid. Six examples of bilateral nodules in the same gland were counted as only one of the pair. The 116 mice happened to have been observed in four groups. Group I consisted of old breeders killed at 20 months; Group II ovariectomised at 20 months and killed at 24; Group III killed at 24 months. Group IV consisted of other breeders which died or were killed at various ages between 12 and 25 months.

TABLE I.—*Number of Times Hyperplastic Nodules Occurred in the Five Pairs of Nipple Regions.*

Groups.	Number of mice with nodules.	Pairs of nipple regions.					Total nipple regions.
		1st.	2nd.	3rd.	4th.	5th.	
I	32	5	21	5	8	7	46
II	19	1	16	2	5	2	26
III	28	1	14	5	9	1	30
IV	37	9	17	4	13	1	44
Totals	116	16	68	16	35	11	146

As will be seen in Table I, nodules were found in the 1st nipple region 16 times, in the 2nd 68 times, in the 3rd 16 times, in the 4th 35 times and in the 5th 11 times. A predilection for the 2nd pair was greater than could have been due to chance and indicates that some localising factor was at work.

These observations appear to have a threefold value. Localisation of a

favouring or predisposing factor may enable one to detect this factor. Attempts to substitute, enhance or eliminate it may be applied to the 2nd and 4th nipple regions. Knowledge of the probable site of the majority of nodules is an aid to finding them in unfixed fresh material. In order to test the potentialities of the nodules it is necessary first to find and transplant them unfixed into young animals of the same substrain or into tissue cultures in order to ensure a longer period of time for observation or experiment. Both 2nd and 4th nipple regions are worth prolonged search.

SUMMARY.

In old breeders of the substrain RIIIb the second pair of nipple regions was almost twice as often the site of hyperplastic nodule formation as the 4th pair and four times as often as any other pair.

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THE LOCAL ACTION OF 20-METHYLCHOLANTHRENE AND SEX HORMONES ON PROSTATIC GRAFTS.

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PREVIOUS studies on the histogenesis of neoplasms arising from homologous subcutaneous grafts of mouse prostatic epithelium impregnated with a carcinogen have indicated that the actively secreting prostatic alveolus is never the focus of malignant change (Horning, 1949, 1952). These experiments suggest that the non-secreting resting alveolar cells in the exhaustive phase of the secretory cycle are more susceptible to the local action of the carcinogen than those at the height of secretory activity. In view of these results, which are in accord with the contention of Haddow (1947) that chemical carcinogens act more readily in cells following depression of cellular activity, experiments were devised to compare the depressant action of oestrogen in combination with the carcinogen with the contrary action of testosterone which stimulates the secretion of prostatic epithelium.

This investigation was further extended in order to ascertain if the inclusion of either the male or female sex hormone together with the carcinogen either retards or accelerates the development of carcinogenesis. For this purpose a series of prostatic grafts was prepared with the carcinogen alone, and others with either oestrogen or testosterone in combination with the carcinogen. Since the success of this method is dependent upon homologous grafting, additional experiments were also made to elucidate problems relating to the graft-host relationship.

TECHNIQUE.

The mouse prostatic epithelium is almost identical in its histology with that of the human (Horning, 1949). Hyperplasia of the prostate gland, however, never occurs spontaneously in rodents, as in man, but a similar condition sometimes arises in mice following prolonged treatment with oestrogens, which induces a pronounced metaplasia of the prostatic epithelium in the dorsal and ventral lobes, involving, as in man, urethral obstruction, retention of urine and hydronephrosis (Horning, 1949).

The method of tumour induction consists in isolating small strips of prostatic epithelium from either the dorsal, ventral and anterior lobes of the prostate gland, impregnating them with crystals of the carcinogen or hormone prior to transplanting them subcutaneously into host mice of the same strain, sex and age, from which the donor tissues were obtained. Details of this method of implantation have previously been described (Horning, 1946, 1947, 1949, 1952).

Male mice belonging to the Strong A and C_3H strains, all approximately

under 12 weeks of age, were selected for these experiments. In some instances pieces of prostatic epithelium were treated with crystals of 20-methylcholanthrene alone, and in others the carcinogen was applied with either stilboestrol or testosterone propionate. Another series of prostatic grafts was prepared and was impregnated with either the male or female sex hormone respectively. Grafts treated with the carcinogen alone or in combination with either of the sex hormones were fixed for histological examination at intervals from 4 to 16 weeks following subcutaneous implantation into host mice. Prostatic implants treated with the hormones alone were obtained for examination at intervals ranging from 12 hours to 6 weeks after grafting.

All grafts fixed for histological examination were fixed either in Zenker-formol or in alcoholic Bouin, and were subsequently stained with haematoxylin and eosin.

RESULTS.

1. *Graft-host relationship.*

The factors involved in the successful survival of homologous grafts growing subcutaneously in host mice will be briefly discussed. It is advantageous that the grafted donor tissue for implantation should be selected from mice of the same strain and age as those of the host animals, and when grafting prostatic epithelium it is particularly beneficial to use male host mice.

Previously it has been shown that grafts transplanted into host mice of indeterminate ancestry have less chance of survival (Horning, 1947, 1952; Pan and Gardner, 1948). In nearly every series of experiments, however, a variable number of prostatic grafts implanted subcutaneously into host mice of the same strain, sex and age as those of the donor fail to survive, and gradually become absorbed by the tissues of the host. Experiments into this aspect of the problem have demonstrated that successful survival of the donor implant is dependent upon a rapid vascularisation of the graft within the tissues of the host mouse, irrespective as to whether the grafts were impregnated with either a carcinogen or sex hormones, or with a combination of both.

Fig. 1 to 4 illustrate the macroscopical appearance of successfully vascularised prostatic grafts growing subcutaneously in both Strain A and C_3H male host mice fixed 6 weeks after implantation. Fig. 2 and 4 are a higher magnification of the same grafts, both of which clearly demonstrate the degree of vascularisation within the tissues of the host mice essential for survival and subsequent growth. A transverse section through one of these grafts (Fig. 4) is shown in Fig. 5, and illustrates the histological relationship between the underlying prostatic graft and the skin of the host-bearing mouse. The prostatic epithelium is healthy and is actively secreting; some of the alveoli are seen distended with prostatic secretion to the point of cystic dilatation. The alveolar epithelium in this particular graft actually shows the various stages in the secretory cycle of the normal prostatic gland.

It is interesting to compare the histology of these successfully vascularised grafts seen in Fig. 1 to 5 with a similar prostatic graft after the same period of implantation, in a strain A mouse, which failed to become vascularised within the host tissues (Fig. 6). The alveoli have completely broken down, there is evidence of a marked lymphocytic infiltration around the periphery of the whole graft, which has become encapsulated, and is in the process of being absorbed by the tissues of the host mouse.

It is relatively easy to detect by palpation non-vascularised grafts, as they are smaller and invariably softer when compared with successfully vascularised prostatic grafts, which are firmer on palpation, larger and invariably cystic in the earlier phases of growth, owing to active secretion of the glandular epithelium.

Fig. 7 is a section through an untreated non-vascularised graft fixed 5½ weeks after implantation, and shows the earlier histological changes which usually accompany the breakdown of the graft. The first histological change seen in grafts after failure to become vascularised is the complete loss of power of the prostatic epithelium to secrete (Fig. 7), also the prostatic fluids, instead of being evenly dispersed throughout the lumina, form distinct concretions similar to those often seen in the human gland. This contrasts with the prostatic secretion seen in successfully vascularised grafts, and also in the normal mouse prostate gland *in situ*, which never forms concretions.

In these early stages of absorption of non-vascularised grafts, the alveolar epithelial cells invariably become detached from the alveolar wall and surround the concretions in a characteristic manner (Fig. 7). Subsequently the alveoli undergo involution, and later the epithelium surrounding the concretions slowly undergoes cellular degeneration and disappears. The prostatic concretions become absorbed by the connective tissues of the host, and this process is accompanied by a pronounced lymphocytic infiltration with final encapsulation of the whole graft as illustrated in Fig. 6.

2. *Grafts implanted with carcinogen alone.*

Because the carcinogen in subcutaneous grafts is placed in direct contact with the living tissue without having to use a solvent, it induces very little foreign body reaction or necrosis. Thus it is possible to study serial sections of the primary grafts, and to trace each invading group of malignant cells back to the individual alveolus from which it had arisen. Histological examination of early neoplasia in some 150 prostatic grafts impregnated with 20-methylcholanthrene alone in previous experiments showed that in no single instance had the actively secreting prostatic epithelium been the focus of malignant change. This process is best appreciated by examining a section through a whole graft of the dorsal prostatic lobe of a Strain A mouse fixed 4½ weeks after implantation, in which adjacent to alveoli lined with a low columnar epithelium and distended with secretions are hyperplastic alveoli, in the exhaustion phase of the secretory cycle (Fig. 8 and 9). The tall columnar epithelial cells, which are characteristic of the exhausted alveoli, are folded and relatively free of secretion probably owing to its absorption by the host tissues, and it is in these alveoli following a phase of mitosis, abnormal cell division and pycnosis that hyperplastic epithelial changes are first detected. These hyperplastic changes in the mouse prostate gland are also accompanied by a pronounced increase in the fibromuscular stroma.

An early neoplastic proliferation in a graft of dorsal prostatic epithelium is seen in Fig. 10. This type of lesion is generally confined to a restricted area in the glandular epithelium of an exhausted alveolus. In this particular implant this was the only malignant focus within the entire graft.

Alveoli distended with secretion and with normal epithelium are seen adjacent to this exhausted alveolus from which this early malignant lesion arose. The individual cells comprising this lesion possessed all the characteristics of malignancy. Some cells were binucleated, others showed abnormal mitosis or con-

tained small nuclei. In every instance the cell boundaries were indistinct (Fig. 10). The proliferating epithelial components in all the early malignant foci break through the alveolar basement membrane, invariably forming tongue-like lesions which rapidly invade the fibromuscular stroma of the host as depicted in Fig. 10. In all prostatic grafts which have grown after treatment with 20-methylcholanthrene, it was found possible to distinguish between three distinct types of early epithelial growth, all of which have been previously described in detail (Horning, 1949).

By studying the cytology and mode of growth in serial sections of these early invasive foci through later phases of development in other similar older grafts, it has been possible to predict the types of tumours which would have subsequently arisen. There is one type which gives rise to a glandular carcinoma, and a second which develops into a squamous growth. Both these types of lesions are easily recognised by their histological character, and both arise from the alveolar epithelium. The third type is the most uncommon variety, characterised by a stratified squamous metaplasia of the epithelium *in situ*, followed by a diffuse marginal growth into the stroma, which clearly arises from the duct epithelium. Occasionally some grafts show two or sometimes three types of proliferating foci within the same implant. When the two former varieties of lesions co-exist in the same primary graft the subsequent tumour becomes a squamous cell carcinoma, which rapidly infiltrates the adeno-carcinomatous areas until all traces of a glandular tumour are lost.

3. *Grafts impregnated with sex hormones alone.*

Stilboestrol.—The rapidity with which the local action of the female sex hormone is capable of depressing the secretory activity of the prostatic epithelium, and inducing squamous metaplasia, is clearly illustrated in Fig. 11. This shows a section of a graft treated with crystals of stilboestrol alone and fixed 4½ weeks after implantation. There is a complete inhibition of secretion after this period of grafting. Every individual alveolus within the graft was entirely devoid of prostatic secretion, and the epithelial cells had undergone marked morphological changes. The typical glandular structure of the epithelium has entirely disappeared, the nuclei are spherical, hypertrophied and frequently hyperchromatic and occasional mitoses are observed. Several alveoli at this stage of treatment may begin to undergo involution (Fig. 12). As early as 26 hours after implantation prostatic grafts treated with stilboestrol show the first histological evidence of secretory inhibition, and the earliest indication of squamous differentiation in the alveolar epithelium is seen in grafts 3 to 5 weeks following transplantation.

Testosterone propionate.—The local action of the male sex hormone on prostatic grafts obtained 4½ weeks after transplantation is shown in Fig. 12. The prostatic epithelium at this period of treatment contrasts greatly with similar grafts treated with the female sex hormone after implantation in host mice for the same period of time. The alveolar epithelium not only maintains its glandular character, but in the majority of grafts testosterone propionate induces a pronounced stimulation of the prostatic secretions, invariably resulting in a cystic dilatation of the alveoli. The glandular epithelial cells of alveoli which are entering into the phase of the secretory cycle contain secretion droplets, and the proximal regions of these cells project into the lumina of the gland. The epithelial

nuclei are oval and normal in appearance. It is characteristic of this form of treatment that numbers of epithelial cells are invariably seen in division (Fig. 12), some exhibiting chromosomal abnormalities in the metaphase. Atypical alveolar formation is sometimes seen in grafts after 7 weeks' implantation, which is apparently due to the manner in which the male sex hormone stimulates glandular epithelium to undergo cell division (Fig. 13).

It would appear that testosterone propionate does not possess such a rapid action on the prostatic epithelium in grafts as compared with stilboestrol, since the first changes are not cytologically manifest until 6 to 8 weeks after subcutaneous transplantation.

4. *Grafts combined with the carcinogen and sex hormones.*

20-methylcholanthrene and testosterone propionate.—Three squamous-celled carcinomas developed from prostatic grafts impregnated with the carcinogen and the male sex hormone in Strain A mice at intervals ranging from 7 to 9 weeks following implantation. One sarcoma arose in a C_3H mouse after 8 weeks (Table I, Group III). As the growth of this tumour was far advanced it was

TABLE I.

Group.	Substances included with the graft.	Strain.	Total no. bearing grafts.	No. which developed tumours.
I	20-emthylcholanthrene	A and C_3H	35	13
II	Methylcholanthrene and stilboestrol	A and C_3H	35	26
III	Methylcholanthrene and testosterone propionate	A and C_3H	35	3

This table is devised to indicate more concisely the grouping of the numbers of tumours which arose from homologous prostatic grafts which had been treated with 20-methylcholanthrene alone, or in combination with either the male or female sex hormone together with the carcinogen.

impossible to ascertain whether it arose from the stroma of the graft or the connective tissues of the host mouse. All the prostatic carcinomas in Group III differed histologically from the growths in Group II (Table I) treated with stilboestrol together with the carcinogen, inasmuch as the alveolar character of the prostate gland persisted. The epithelial components lining the lumina of the alveoli retained their glandular structure with elongated nuclei and a deeply staining cytoplasm typical of the normal prostatic epithelium (Fig. 14). There was, however, a total absence of secretion both in the alveolar epithelium and the lumina of the tumour growth (Fig. 14). Mitotic activity was more conspicuous in the columnar cells lining the alveoli than in the squamous cells at the periphery which had undergone metaplastic changes. All these prostatic carcinomas exhibited a considerable degree of keratinisation which was entirely restricted to the peripheral regions of the alveoli. Only one of the tumours showed concentric nests of cells with keratin pearls and prickle formation.

20-methylcholanthrene and stilboestrol.—Unlike the grafts in Group I (Table I) combined with the carcinogen alone, in which both glandular and squamous-celled carcinomas were induced, only squamous tumours developed following treatment with the carcinogen and female sex hormone. These squamous-celled growths consisted of two distinct varieties. The less common type was characterised by a stratified squamous metaplasia of the duct epithelium, which

was later followed by a diffuse marginal growth into the stroma of the graft sometimes seen in grafts treated with the carcinogen alone. The more common type of squamous growth invariably arose from foci in the alveolar wall which had previously undergone squamous metaplasia. The proliferating cells rapidly infiltrate the graft and finally invade the connective tissues of the host (Fig. 15). In three grafts out of the 35 examined both types of squamous growth persisted.

Grafts treated with the carcinogen together with stilboestrol do not, as a rule, form pseudo-alveoli. In this respect they differ from similar grafts combined with testosterone propionate and methylcholanthrene. These squamous growths invariably formed intercellular fibrils of " prickle " cells followed by keratinisation. The differences in histological structure between prostatic tumours derived from grafts treated with the carcinogen together with either the male or female sex hormone can best be appreciated by comparing Fig. 14 and 15. It was of interest to note that the second group of prostatic grafts treated with this particular sex hormone and the carcinogen produced 26 tumours from a total of 35 grafts, whilst similar grafts over the same period of implantation as seen in Groups I and III produced only 13 and 3 tumours respectively. Not only did the presence of stilboestrol with 20-methylcholanthrene induce the greater numbers of tumours, but grafts when treated with this combination of compounds gave rise to small palpable nodules at an earlier period after transplantation ; in many instances these were detected as early as 6 weeks after grafting. These experiments indicate that the presence of the female sex hormone when in combination with the carcinogen accelerates the development of carcinogenesis.

5. *Examination of pituitary and adrenal glands and gonads from host mice bearing grafts.*

Examination of pituitaries from mice bearing grafts from all three groups of rodents treated either with the carcinogen alone or in combination with either of the sex hormones revealed no abnormal histological changes, with the exception of excessive amounts of colloid seen in the clefts of pituitaries from mice treated with stilboestrol and the carcinogen. There was no diminution of acidophiles or increase in chromophobe cells following implantation of the stilboestrol-treated grafts. Neither did the adrenal cortex reveal any histological abnormalities resulting from this treatment. The ovaries also appeared normal and the testes showed no inhibition of spermatogenesis.

DISCUSSION.

One of the several advantages arising from homologous grafting in the presence of a carcinogen is that the tumours growing under the skin are palpable and can be readily obtained at any required phase of growth. Likewise it also permits an early cytological study of neoplastic and other changes in individual grafts. More important still, this procedure renders it possible to observe the local and direct action of numerous chemical compounds other than carcinogens upon individual cells within a given graft.

This method of rapidly inducing tumours from subcutaneous homologous grafts impregnated with a carcinogen, described by Horning (1947), has since been successfully employed by Pan and Gardner (1948), and Hughes (1949*a*, 1949*b*). The factors involved in the successful survival of subcutaneous grafts

of both lung and prostatic tissues have been previously described by the author (1949, 1952). In mice the survival of an homologous graft seems to be dependent upon the use of a closely inbred strain, since stock mice of indeterminate ancestry do not easily tolerate grafts of this kind. Pan and Gardner (1948) found an increased susceptibility to tumour formation from grafted uterine epithelium in pure line mice as compared with hybrids.

Recent experiments (Horning, 1952) have further indicated that the age of the donor providing the graft, and the age of the host mouse into which the grafts are implanted, also have an important bearing on the problem of graft survival. Other factors such as the reaction beween the graft and the host mouse play an important rôle. It has been previously reported that more difficulty has been experienced in obtaining malignant tumours from lung grafts impregnated with 20-methylcholanthrene than with similar grafts of other tissues. It was interesting to note that tissues which are normally under hormonal influence are much better tolerated as grafts in host mice of the same sex and strain than tissues which are to a greater extent (such as the lung) independent of hormone action (Horning, 1952). Nevertheless, a certain number of prostatic grafts, when implanted subcutaneously into host mice of the same strain as those of the donor, fail to survive irrespective as to whether the grafts were impregnated either with the carcinogen alone, or in combination with either the male or female sex hormone. The present experiments on the reaction between the implanted prostatic graft and the host bearing mouse have shown conclusively that the survival of the donor graft is dependent upon a rapid vascularisation within the subcutaneous tissues of the host mouse. The reason, however, why some grafts fail to become vascularised still remains obscure.

The influence of steroid hormones on the behaviour and growth of prostatic cancer has been the subject of intensive investigation. One of the difficulties in interpreting the relationship of sex hormones to carcinogenesis is that most experiments which have been devised are complicated by the fact that the endocrine balance or metabolism of the treated animals has been altered by the administration of hormones. These experiments have shown, however, that although the combination of steroid hormones in subcutaneous implants induces rapid changes as the result of a direct and local action on the grafted tissue itself, it does not appear to influence either the anterior hypophysis, adrenals or the gonads of the host animals bearing the grafts. It can therefore be assumed that the impregnation of tissues with hormones prior to transplantation into host mice yields more decisive results, because the hormone by being placed in direct contact with the grafted tissue has a local and direct action.

The reason why prostatic tumours arise more rapidly and in greater numbers from grafts treated with the female sex hormone plus the carcinogen than with the carcinogen alone or in combination with the male sex hormone is of interest when considering Haddow's (1947) contention that chemical carcinogens act more readily on cells following depression of cellular activity. Previous experiments have shown two interesting facts, namely that the focus of malignant change is restricted to the prostatic epithelium of the alveoli in the exhausted phase of the secretory cycle, and also that oestrogens in mice are capable of inhibiting the secretory activity of the prostatic epithelium 24 hours following administration (Horning, 1947, 1949). These previous findings, together with the results obtained in these experiments, strongly suggest that the depressant

action of stilboestrol on the secretory processes of the alveolar epithelium renders these cells more susceptible to the influence of 20-methylcholanthrene.

SUMMARY.

(1) The factors involved in obtaining successful subcutaneous homologous grafts from the prostatic epithelium of mice have been discussed.

(2) Chemical carcinogens act more readily on cells following depression of cellular activity. In grafts treated with the carcinogen alone, the focus of malignant change is restricted to the epithelium of alveoli which have entered into the phase of secretory exhaustion.

(3) Tumours arise more rapidly and in greater numbers in prostatic grafts treated with the female sex hormone plus the carcinogen than they do in similar grafts which have been impregnated with the male sex hormone combined with the carcinogen, or in grafts treated with the carcinogen alone.

(4) It appears that stilboestrol by inhibiting the secretion of the prostatic epithelial cells renders them more susceptible to the action of the carcinogen.

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EXPLANATION OF PLATES.

FIG. 1.—The skin of a C_3H male mouse is cut and pinned back so as to expose an untreated homologous prostatic graft which had been growing subcutaneously for 6 weeks. The grafted tissue was obtained from the dorsal prostate of a C_3H mouse of the same age and sex as that of the host rodent. × 2.

FIG. 2.—A more highly magnified macroscopic view of the graft seen in Fig. 1, showing the degree of vascularisation of the implant. Note also the convolutions on the surface of the graft, which shows evidence of cystic dilatation. × 8.

FIG. 3.—An homologous prostatic graft is seen growing subcutaneously in a Strain A host male mouse. The prostatic implant was obtained from the ventral prostatic lobe of a Strain A mouse of the same strain and age as that of the host rodent. × 2.

FIG. 4.—Same as that seen in Fig. 3, only more highly magnified to demonstrate the degree of vascularisation within the tissues of the host mouse essential for survival and subsequent growth. × 8.

FIG. 5.—A transverse section of prostatic graft, which is seen macroscopically in Fig. 3 and 4. This photomicrograph clearly illustrates the histological relationship between the underlying homologous graft to that of the skin of the host mouse. The prostatic epithelium is actively secreting, and many of the alveoli are distended with prostatic secretions. × 4.

FIG. 6.—Transverse section through an homologous prostatic graft which failed to become vascularised, fixed 6½ weeks after subcutaneous implantation. The relationship of the grafted prostate to that of the skin of the host rodent is clearly shown. The prostatic

alveoli have completely broken down; there is also evidence of lymphatic infiltration. The whole graft has been encapsulated and is slowly being absorbed by the tissues of the host mouse. The graft was obtained from the dorsal prostatic lobe of a Strain A mouse, and implanted subcutaneously in a mouse of the same sex, age and strain as that of the host. × 4.

FIG. 7.—Section through an untreated homologous prostatic graft fixed 5½ weeks after implantation showing the earlier histological changes which usually accompany the breakdown in a graft following failure to become vascularised. Note the abnormal increase in the fibro-muscular stroma of the graft together with the loss of power of glandular epithelium to secrete. The prostatic fluids have formed distinct concretions, and the alveolar epithelium is shown here becoming detached from the alveolar wall and mobilising around the concretions within the prostatic lumina. × 95.

FIG. 8.—Section through a whole homologous graft obtained from the dorsal prostatic lobe impregnated with 20-methylcholanthrene and fixed after 4½ weeks subcutaneous implantation into a Strain A host mouse. Adjacent to alveoli lined with low columnar epithelium and distended with secretion are hyperplastic alveoli, in the exhaustion phase of the secretory cycle. × 15.

FIG. 9.—Higher magnification of area marked in Fig. 8, showing the alveoli distended with secretion lined by low columnar epithelium adjacent to which is the hyperplastic alveolar epithelium with lumina devoid of secretion. These early hyperplastic changes in the alveolar epithelium are a response to the carcinogen. In some regions of the graft the glandular epithelium contains patches which are 7 to 9 cells in depth. × 90.

FIG. 10.—Early neoplastic proliferation in an homologous prostatic graft, growing in a Strain A mouse. This graft was impregnated with 20-methylcholanthrene and fixed 9½ weeks after subcutaneous implantation. In this particular graft this was the only malignant focus within the entire implant. As is clearly seen, it arose from the epithelium in an exhausted alveolus. Note the normal appearance of glandular epithelium lining adjacent alveoli distended with secretion. This early malignant tongue-like formation of cells (indicated by arrows) is invading the fibro-muscular stroma of the graft. × 240.

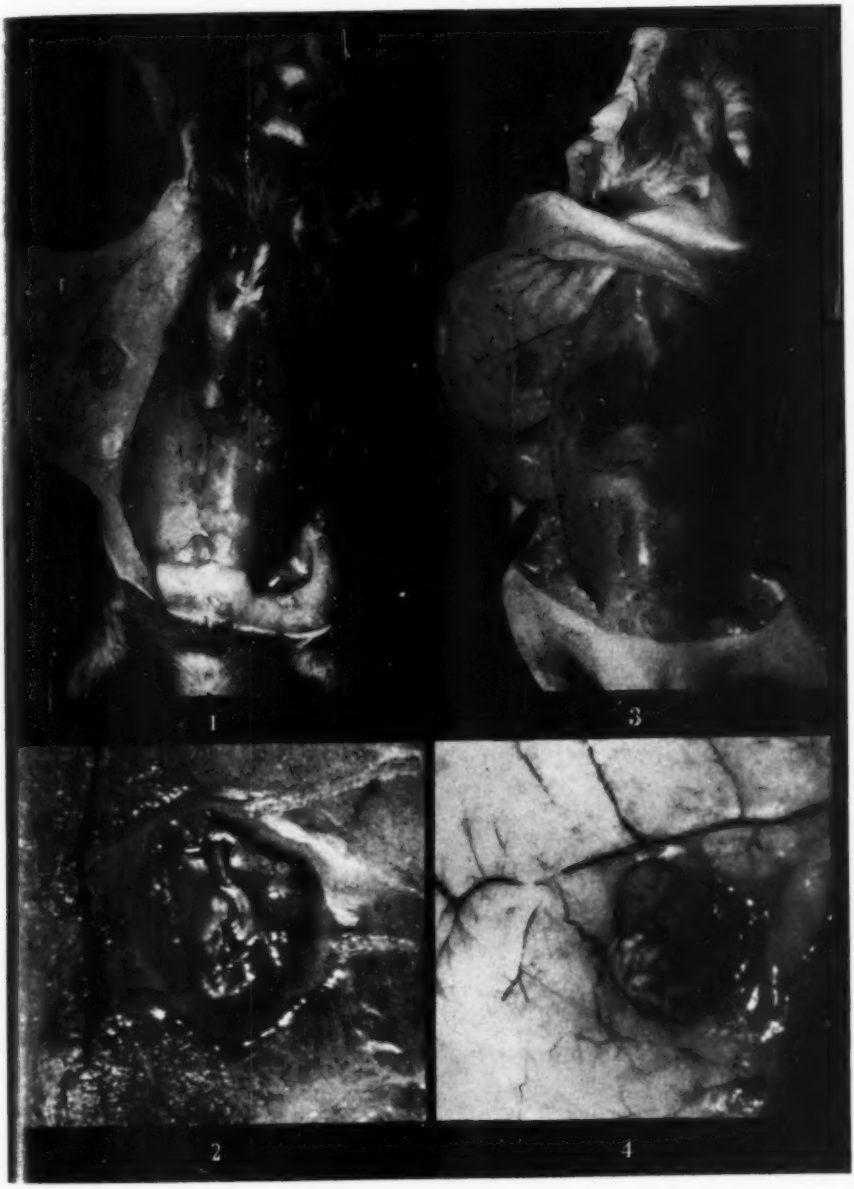
FIG. 11.—Homologous prostatic graft impregnated with diethylstilboestrol alone fixed 4½ weeks after subcutaneous implantation in a Strain A host mouse. Note the complete inhibition of secretion. The graft is entirely devoid of prostatic secretions, and the typical glandular structure of the epithelium has disappeared. × 530.

FIG. 12.—Homologous prostatic graft impregnated with crystals of testosterone propionate alone, fixed 4½ weeks after subcutaneous implantation. Grafts thus treated contrast greatly with similar grafts treated for similar periods of time with the female sex hormone (Fig. 11). The alveolar epithelium maintains its glandular character, as the male sex hormone induces stimulation of the prostatic secretions, invariably resulting in cystic dilatation of the alveoli. Note several epithelial cells in division. × 400.

FIG. 13.—Prostatic graft combined with testosterone propionate alone, fixed 7 weeks after subcutaneous implantation in a C_3H male mouse showing the stimulating action of the male sex hormone on the prostatic epithelium. Two cells of the glandular epithelium are seen in division, and another in an atypical alveolus near the right bottom corner of the photomicrograph. × 400.

FIG. 14.—Prostatic carcinoma which arose from a graft of prostatic epithelium impregnated with both methylcholanthrene and testosterone propionate. Prostatic carcinomas in this group (Table I, Group III) differ histologically from those tumours induced in grafts treated with the carcinogen plus the female sex hormone. Compare Fig. 14 and 15. Although this carcinoma is a squamous celled growth the alveolar formation of the neoplasm persists. × 200.

FIG. 15.—Early squamous-celled carcinoma which arose from a graft of prostatic epithelium treated with the carcinogen together with stilboestrol (compare with Fig. 14). × 120.



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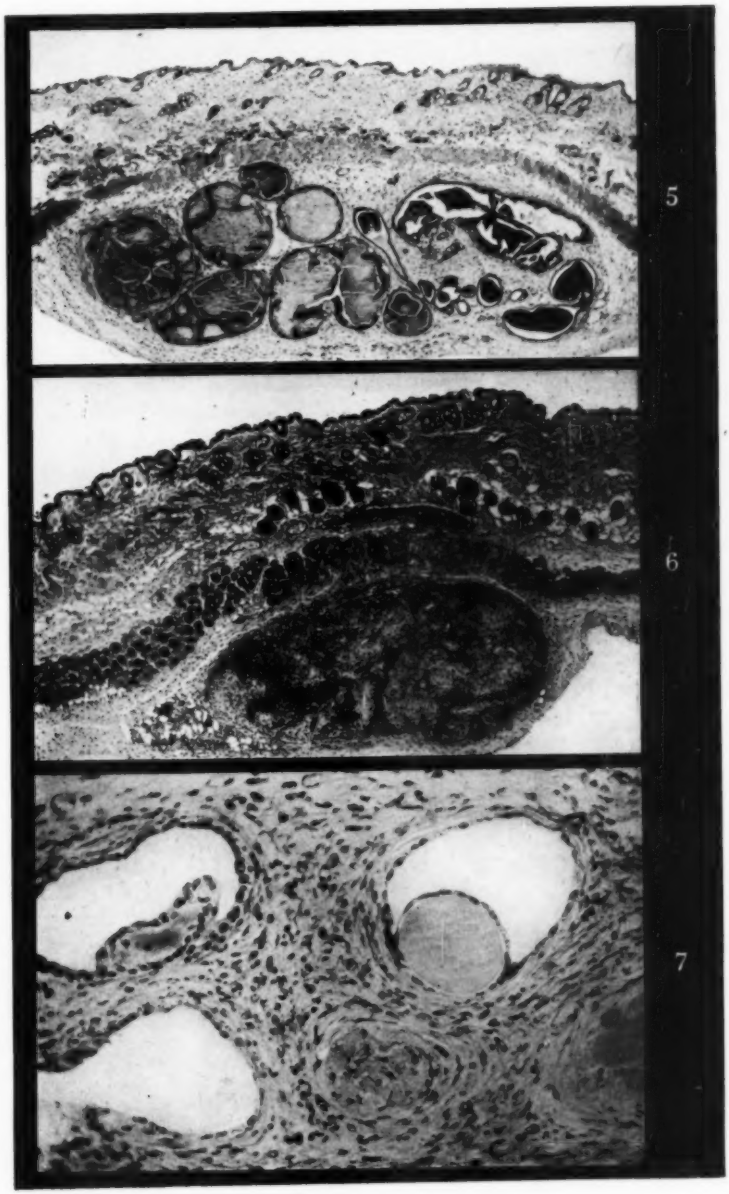

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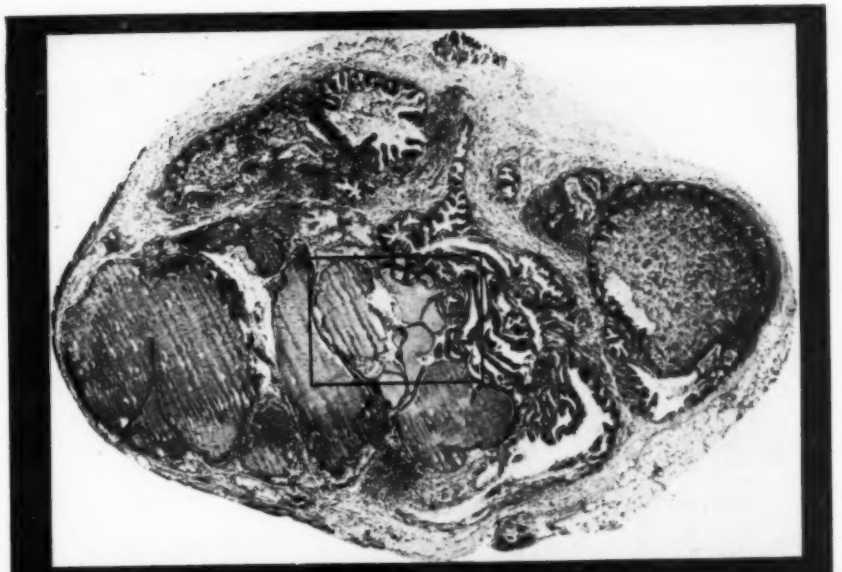

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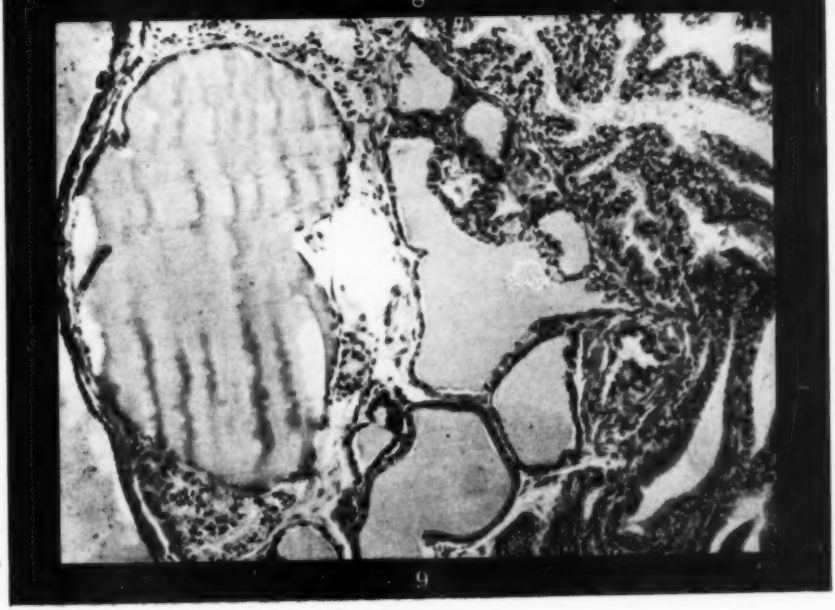

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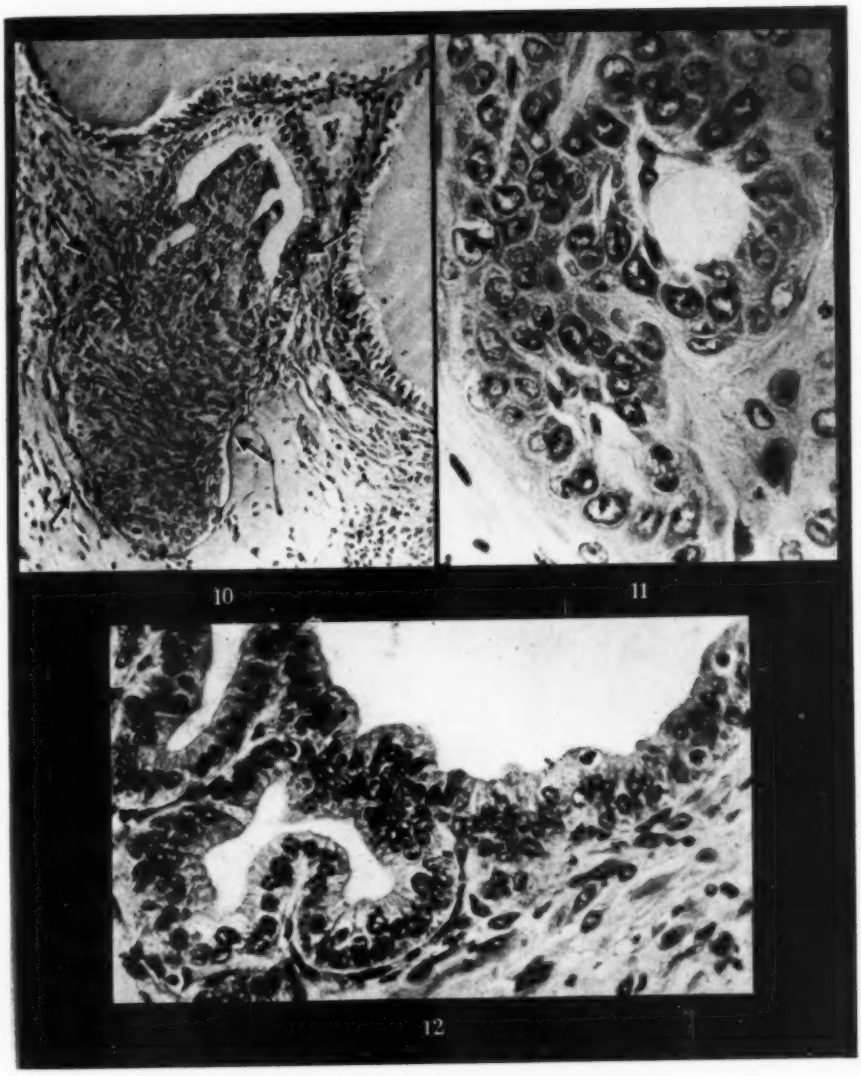

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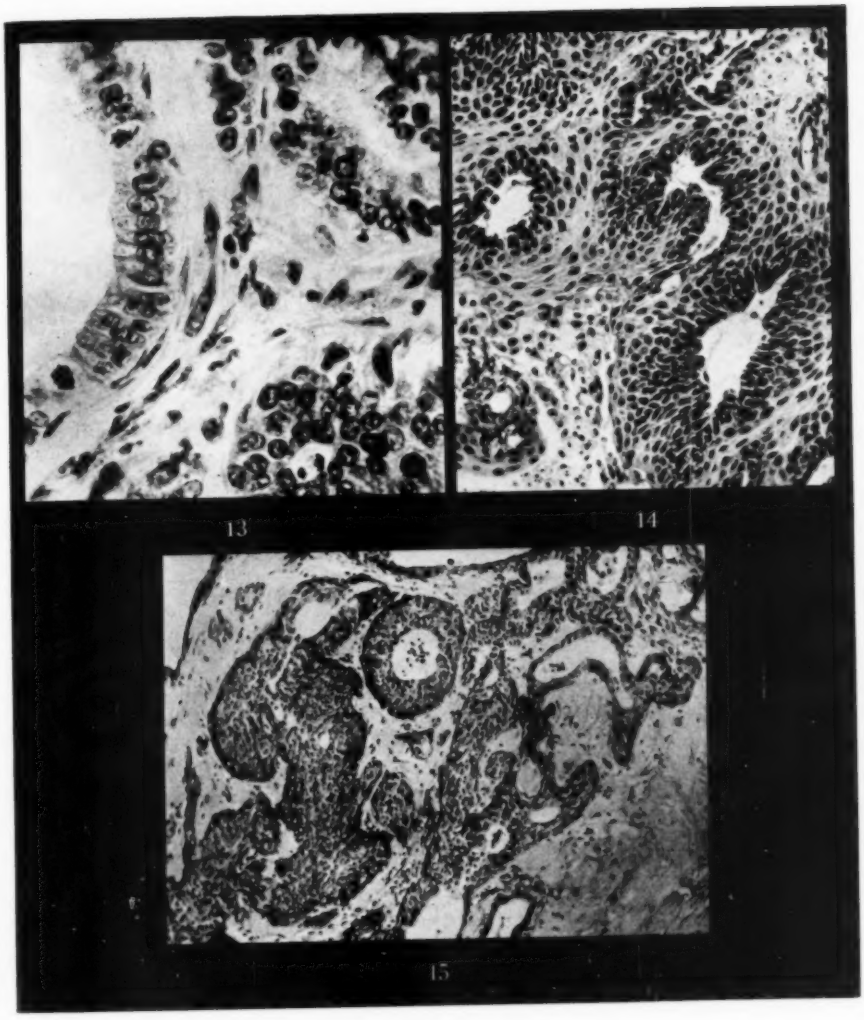

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THE CARCINOGENIC ACTIVITY OF N-MONOMETHYL- AND N-DIMETHYL-2-AMINOFLUORENE.

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ACETYLAMINOFLUORENE (A.A.F.) has been found to induce tumours in many organs of the rat. While this versatility is of advantage in certain experiments, in others it is a handicap, especially when the induction of tumours in one particular organ is the goal of an investigation. Thus the great susceptibility of the liver of the male rat to A.A.F. makes it extremely difficult to obtain neoplasms of other organs without at the same time inducing hepatomas. The mono- and dimethyl derivatives of 2-aminofluorene were tested in order to ascertain whether methylation of the amino group modifies carcinogenic activity, especially in regard to the liver of the male rat.

METHODS.

N-monomethyl-2-aminofluorene (M.A.) : 2-acetylaminofluorene (4·5 g.) is dissolved in boiling xylene (200 ml.) and an excess of metallic Na (1 g.) is gradually added. The mixture is refluxed for 5 hours and then left to cool to about 60°. CH_3I (2 ml.) is added and the mixture refluxed for 1 hour. After standing overnight the precipitate is removed and the filtrate brought to dryness by distillation *in vacuo*. The residue is dissolved in ethanol (10 ml.) and refluxed for 1 hour with 18 per cent HCl (40 ml.). On cooling, the hydrochloride of M.A. crystallizes out and is recrystallized from 18 per cent HCl with addition of charcoal. M.A. is obtained by adding methylamine to the solution of the hydrochloride in 60 per cent ethanol. After recrystallization from 50 per cent ethanol M.P. was 74°.

N-dimethyl-aminofluorene (D.A.) : D.A. is obtained by methylation of 2-aminofluorene with dimethylsulphate in alkaline solution. After two recrystallizations from methanol M.P. was 182°.

Two different strains of rats were used, in Experiment I piebald rats of the Sheffield strain and in Experiment II Wistar rats of the New Zealand colony. They were 6 to 8 weeks old at the beginning of the experiment. The piebald rats received 4·0 mg. of D.A. or 3·5 mg. of M.A. daily with their diet (Bielschowsky, 1944) for 25 weeks. In Experiment II the ordinary stock diet (Hall, 1948) was given and the carcinogens, dissolved in peanut oil, were administered by stomach-tube. The rats received 3 doses of 3·8 mg. of D.A., 3·5 mg. of M.A. or 2 mg. of A.A.F. per week ; 3·8 mg. of D.A. and 3·5 mg. of M.A. are equivalent to 4 mg. of A.A.F., so that in Experiment II the rats treated with D.A. and M.A. received

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twice as much of the methyl compounds as of A.A.F. given to the controls. Ninety doses were given to each group. The animals were sacrificed as soon as the presence of a tumour was detected. The survivors of Experiment I were killed in the 63rd and those of Experiment II in the 52nd week of the experiment.

RESULTS.

Table I summarizes the results of Experiment I. For comparison the tumours obtained by feeding 4 mg. of A.A.F. for 25 weeks to rats of the same strain,

TABLE I.—*Piebald Rats.*

	Dimethyl-2-aminofluorene.		Monomethyl-2-aminofluorene.		Acetyl-2-aminofluorene.	
Sex	♂	♀	♂	♀	♂	♀
Total numbers	5	5	5	5	13	25
Animals with—						
Hepatomas	1	—	4	—	10	10
Cancers of meatus acousticus externus	—	—	1	—	10	20
Cancers of intestine	—	2	1	—	6	16
Tumours of lung	—	2	2	2	1	2
Leukaemias	—	—	—	—	1	—
Mammary cancers	—	—	—	—	—	1
Cancers of eyelid	—	—	—	—	—	1

receiving the same diet, have been included (Bielschowsky, 1946). All the males receiving A.A.F. had palpable tumours before the end of the 33rd, and all but 2 of the females before the end of the 42nd week. The rats treated with D.A. were apparently still free of neoplastic lesions in the 63rd week of the experiment, all the tumours induced by D.A. being discovered at the post-mortem examination. In the group receiving M.A. the first tumour, a hepatoma, was found in the 54th week, and the second, a cancer of the meatus acousticus externus, 3 weeks later. Both occurred in males. The other neoplastic lesions induced by M.A. were found when the experiment was terminated 6 weeks later.

Table II gives the results obtained by the administration of D.A., M.A. and A.A.F. by stomach-tube to Wistar rats. In Experiment II palpable tumours

TABLE II.—*Wistar Rats.*

	Dimethyl-2-aminofluorene.		Monomethyl-2-aminofluorene.		Acetyl-2-aminofluorene.	
Sex	♂	♀	♂	♀	♂	♀
Total numbers	5	5	5	5	5	5
Animals with—						
Hepatomas	—	—	2	—	4	1
Cancers of meatus acousticus externus	2	1	3	3	2	1
Tumours of lung	—	—	2	1	2	—
Mammary cancers	—	3	—	4	—	3

appeared in the females of all groups at a rather early date. They were tumours of the breast. The first mammary cancer was found in a female of the control group (A.A.F.) 20 weeks after the administration of the carcinogen had been started. By the 24th week neoplasms of the breast gland were present also in the two other groups. Cancers of the meatus acousticus externus made their appearance in rats treated with the methyl compounds from the 34th week onwards, whereas in the A.A.F. group the first was seen in the 25th week. Palpable liver tumours, however, occurred only in the latter group after the 33rd week of the experiment.

The total yield of cancers induced by D.A. was higher in the second experiment, but, as in Experiment I, the livers were remarkably little affected. Only 3 of the males but none of the females showed macroscopically recognizable lesions. These consisted of a few minute cysts. Microscopically, besides a few rather inactive looking benign cystic cholangiomata, a slight increase in periportal tissue together with small foci of highly vacuolated liver cells were found. M.A. induced two hepatomas, definite signs of liver damage and fairly widespread cystic cholangiomata in 2 other males of this group, while 2 of the females showed a few minute cysts in the liver. No animal of the A.A.F. group was completely free from neoplastic changes of the liver. Four hepatomas were found in the males and one in the females, cystic cholangiomata being present in all.

To summarize, the genetic background determined to a large degree the site of tumour development. Thus breast tumours appeared in a much higher incidence in the Wistar rats than in the piebalds, in which cancers of the small intestine were readily induced. Nevertheless in both strains a striking difference in the response of the liver to the three compounds was apparent. In the first experiment 700 mg. of A.A.F. or of D.A. and 612·5 mg. of M.A. were given, while in the second the total dose administered amounted to about half of the methyl compounds and to approximately a quarter in the case of A.A.F. Yet in both experiments A.A.F. induced more malignant hepatomas than either of the methyl compounds. No striking differences were found in the incidence of neoplasms of other organs.

DISCUSSION.

The hypothesis that the acetyl group of A.A.F. is readily split off *in vivo* and therefore can be only of minor importance for the carcinogenic activity has been substantiated by Weisburger, Weisburger and Morris (1950), who showed that after feeding ω-C^{14}-2-acetylaminofluorene radioactivity appeared in the breath within 6 hours.

Ray and Argus (1951) found the toluene-sulphonic ester of 2-aminofluorene to be non-carcinogenic for the rat, only minute amounts of this ester being hydrolysed in the body. Morris, Dubnik, Dunn and Johnson (1947), studying the action of 2-nitro, 2-amino, mono-acetyl and diacetyl-2-aminofluorene, reported that the acetyl compounds induced the majority of liver tumours observed, when the substances were given *per os*.

Why the acetylated aminofluorenes have such an affinity for the liver, whereas M.A. and especially D.A. are less carcinogenic for this organ, remains to be explained. The results of Experiment I suggested that the low incidence of hepatomas induced by D.A. was a sign of its relatively low carcinogenic potency, as the total tumour yield was rather small. However, in the second experiment

the dimethyl compound induced breast tumours in the females of the Wistar strain nearly as readily as M.A. It seems, therefore, doubtful whether this explanation is entirely adequate.

The development of breast tumours in animals whose livers appear macroscopically and microscopically normal was seen not only in rats treated with D.A. but also in females treated with A.A.F. for 13 weeks (unpublished results). Therefore liver damage does not seem to be a prerequisite for the induction of mammary cancers by aminofluorene derivatives.

SUMMARY.

The carcinogenic activity of N-dimethyl- and of N-monomethyl-2-aminofluorene has been compared with that of N-acetyl-2-aminofluorene.

The dimethyl compound was found to be considerably less carcinogenic for the liver of rats than either the monomethyl compound or the acetyl ester (A.A.F.).

The incidence of mammary cancers in females of the Wistar strain treated with N-dimethyl-, N-monomethyl- or N-acetyl-aminofluorene was of a similar order of magnitude.

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THE USE OF CELLULOSE FIBRES AND FILMS IN THE CULTURING OF TISSUES.

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THIS investigation into the growth of tissues *in vitro* with added cellulose was the outcome of dissatisfaction with present-day methods when applied to certain problems. Most living cells when grown *in vitro* as hanging-drop preparations rely upon the fibrin network for support. The mesh consists of fine threads of protein (Fig. 1), and the cells under certain circumstances, particularly if they are malignant, metabolise this protein and so lose their support.

The paper is concerned with the provision of an alternative network which would not be digested by the cultures. To this end specimens of cellulose in various forms were incorporated in tissue cultures grown by the hanging-drop method.

METHODS OF CULTURE.

Three different preparations were used when testing the samples of cellulose :

1. Chick fibroblasts grown on plasma clots consisting of 1 drop of hen plasma and 1 drop of 25 per cent chick embryo extract were used to test the cellulose for toxicity. The cellulose was added either as films or fibres. Films were merely laid on top of the clot. Fibres were suspended in a drop of saline and added to the medium ; a firm clot was still produced (Fig. 2 and 3).

2. Chick fibroblasts were grown in a liquid medium of mouse serum and chick embryo extract with added cellulose. Since there is no fibrin network, the cells rely wholly upon the cellulose and coverslip for support. Films were placed either under or over the medium. Fibres were added as a suspension to the medium or alternatively were dried on to the coverslip, the culture placed directly on them and a drop of medium spread over the whole (Fig. 4).

3. Cultures of mouse mammary adenocarcinoma from C3H, ABC and A strains were grown in one of the following three media depending on the type of clot it was desired to study. For all such cultures the double coverslip technique of Maximow was used :

(1) 1 drop of hen plasma + 1 drop of chick embryo extract (firm clot).

(2) 1 drop of mouse serum + 1 drop of chick embryo extract (fluid medium).

(3) 1 drop containing mouse serum and hen plasma in equal amounts, + 1 drop of chick embryo extract (soft clot).

The cellulose was incorporated as described for previous preparations.

A total of 17 samples of cellulose prepared in different ways were screened for their suitability for tissue culture work. The following criteria were used, and these eliminated 14 of the samples, leaving 3 which could be said to fulfil all the requirements.

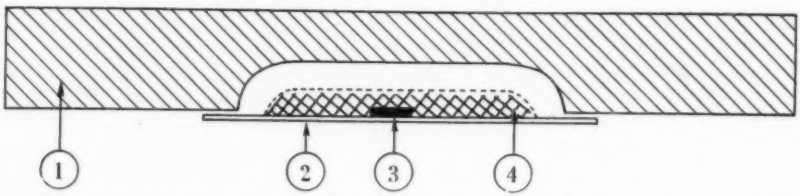

FIG. 2.—Diagram to show the method of incorporating fibres in tissue cultures : (1) depression slide ; (2) coverslip ; (3) culture ; (4) medium containing suspension of fibres.

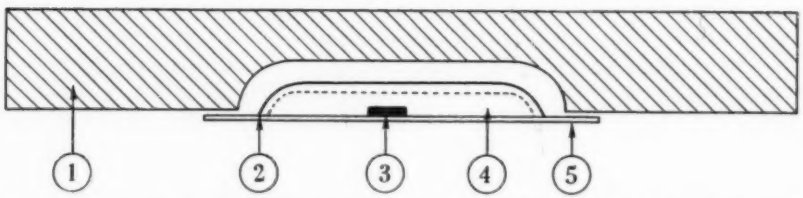

FIG. 3.—Diagram to show the position of Viscose film when used with tissue cultures : (1) depression slide ; (2) Viscose film ; (3) culture ; (4) medium ; (5) coverslip.

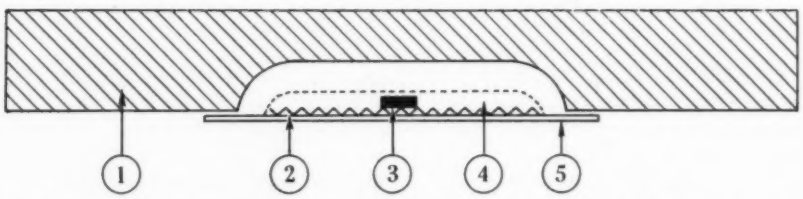

FIG. 4.—Diagram to show the relative position of fibres and culture when fibres are dried on to the coverslip : (1) depression slide ; (2) fibres dried on to the coverslip ; (3) culture ; (4) medium ; (5) coverslip.

Toxicity.

All the samples were tested by adding them to classical fibroblast cultures grown in clots as described above ; eight of the samples were regarded as intrinsically toxic in that they adversely affected growth. These included cellulose precipitated from solution in Triton B, in cuprammonium, in hydrochloric or sulphuric acid. Cellulose acetate films, whether prepared on mercury or directly on coverslips, were also toxic, as were " Aerogel " films prepared from cellophane film.

Ease of handling.

It is important that the fibres should spread evenly in the medium, since a tendency to clump will mean that on further transplantation some of the old medium is carried over and will interfere with subsequent growth. Clumping also adds to the difficulties of microscopic examination. The following were found unsuitable for these reasons :

(i) Cotton broken up by treatment either in a Waring Blendor or in a paper beater and Hurrell mill.
(ii) Bemberg rayon treated in a Waring Blendor.
(iii) Fortisan rayon hydrolysed from cellulose acetate and broken up in a Waring Blendor.

Cellulose as films should cut easily with a cataract knife during subcultivation. Films produced by the *Acetobacter acetiginum* and prepared by the method of Barsha and Hibbert (1934) were discarded as being too tough.

Staining and microscopical qualities.

Samples were subjected to various staining techniques, including Giemsa, Feulgen and Erlich's haematoxylin. In all cases the stain was taken up lightly by the cellulose and did not interfere with subsequent examination. Siraceta rayon treated in a Waring Blendor, however, swelled up during staining by Giemsa method and obscured the field.

The following 3 forms of cellulose were found to be satisfactory on the above tests and all proved to be of value in culturing tissues by the hanging-drop method :

1. *Cotton treated in a colloid mill.*

This was prepared from cotton velvet shearings suspended in water and passed through a colloid mill 6 times in succession. After each passage only the finer fraction was slected for further milling. The form of these fibres is shown in Fig. 5.

2. *Durafil treated in a Nelco homogeniser.*

Durafil (high tenacity Viscose) was also suspended in water and treated for 5 hours at maximum speeds. The same degree of disintegration could probably be done in a shorter time by a Waring Blendor. An electron microscope photograph (Fig. 6) shows the variety in size of these fibres, which may be compared with the fibres in a fibrin clot (Fig. 1). From the technical standpoint both types of cellulose spread easily and evenly on the coverslip and present no difficulties in the subsequent transplantations.

Similar sterilisation methods were applicable to the 2 types. If they were required as a suspension in saline they were autoclaved for 20 minutes at 18 lb./square inch pressure. Otherwise the fibres were suspended in distilled water, a drop was placed on each coverslip and allowed to dry in a low temperature oven. When dry the coverslips were sterilised by dry heat at 150° C. for one hour.

The sterilised suspensions of both types of fibre were found useful as additions to the plasma clot in the cultivation of malignant cells where liquefaction presented a problem. Fibres dried on to the coverslip were of real value in the cultivation of cells either embryonic or malignant in a liquid medium. For this purpose the tissue fragment was placed directly upon the dried fibres and the liquid medium added. The fibres trap the medium satisfactorily and also support the growing cells. Fig. 5 shows the compact growth of mouse mammary carcinoma obtained in the presence of fibres of colloid milled cotton; as a contrast the uneven growth of cells in the absence of fibres is shown in Fig. 7. The growth of tumour cells with added Durafil may be seen in Fig. 8.

3. *Viscose cellulose films.*

Electron microscope photographs of these films reveal a homogeneous surface with no submicroscopic structure visible at a magnification of 20,000.

Preparation.—Viscose containing 7 per cent cellulose and 6 per cent NaOH was allowed to stand at room temperature for several weeks until it coagulated to a stiff gel. The coagulated gel was cut into discs 30μ thick on a microtome and immersed in distilled water. The discs were washed free from alkali with distilled water by repeated decantation. They were then desulphurised by treating with 1·5 per cent ammonium sulphide solution at 45° C. for 5 minutes, washed with distilled water until free from ammonium ion (tested by Nessler's reagent) and sulphide ion (tested by sodium nitro-prusside). They were then bleached 15 minutes at room temperature in sodium hypochlorite solution containing 0·1 per cent available chlorine, and finally washed with distilled water until free from chloride ion (tested by silver nitrate). They were stored under distilled water.

The films were autoclaved in distilled water at 18 lb./square inch pressure, and before use were rinsed in sterile Tyrode solution, excess moisture being removed with sterile filter-paper.

Cultures were placed directly on the coverslip, a drop of mixed serum and embryonic extract spread over them and the Viscose film then applied. The culture with its liquid medium was thus sandwiched between coverslip and Viscose film (Fig. 3). The culture usually adhered to the glass surface, and on washing or fixation the film floated off.

The film is easily cut with a cataract knife when the tissue is subcultured. With a thickness of 30μ the total thickness of the preparation was considerably less than can be achieved with the double coverslip method of Maximow. Films less than 30μ in thickness, although advantageous from the microscopic standpoint, are insufficiently rigid for easy handling.

The observation of living cultures with the film *in situ* is adequate for a cursory examination (Fig. 9), but cellular detail is less clear than when fibres are employed; however, the examination of stained cultures is unimpeded, since the film floats off on fixation (Fig. 10 and 11).

Mitotic counts have been carried out to compare the rate of cell division in plasma cultures without cellulose with that in cultures grown in serum with Viscose film. No difference was found in the percentage counts for the two treatments. The comparison serves to show that the abundant outgrowth obtained with the addition of cellulose is due to cell division as well as to outwandering of the cells of the explant.

Viscose film is probably superior to fibres when a liquid medium is employed ; fibres are more useful in offsetting the effects of liquefaction in a solid medium.

DISCUSSION.

It is felt that the addition of certain forms of cellulose to tissue cultures may be a real gain in many branches of work, particularly those employing malignant cells or in which there is a need for a liquid medium. This latter necessity arises particularly when it is desired to test the addition of a chemical substance to the cells. While it is true that unknown factors of absorption and adsorption may then be introduced with the cellulose, the same factors are operative when a fibrin meshwork is present and have to be considered even for the glass coverslip.

The support given to the cells is not the only reason for the improved growth, which may depend also on the greater amount of medium that can be enmeshed and made available to the cells. Probably any of the forms of cellulose that have been described as successful are equally suitable for many purposes. For stained preparations Viscose has the great advantage of parting from the culture on fixation ; for phase contrast microscopy of the living cell one of the fibres, either colloid milled cotton or Durafil, would be preferable.

Other recent work concerned with replacing the plasma clot is that of Earle and his co-workers. They have found that perforated cellophane is an advantageous addition to flask cultures and hanging-drop preparations (Evans and Earle, 1947 ; Earle and Evans, 1949 ; Schilling, Earle and Evans, 1950 ; Earle, Evans and Schilling, 1950). In our hands perforated cellophane, kindly supplied by Dr. W. R. Earle, was very similar in effect to Viscose, but the outgrowth obtained with Viscose seemed slightly greater.

Glass wool has been used for the same purpose by Warner, Hanawalt and Bischoff (1949), who used it with the roller tube method. The disadvantages quoted by the authors were that adsorption on to the glass remained a problem and that subcultivation presented difficulties. We have not had the opportunity of applying their technique to hanging-drop preparations for comparison with cellulose.

SUMMARY.

1. A variety of forms of cellulose have been incorporated in hanging-drop preparations of normal and malignant cells in tissue culture.

2. Viscose film, colloid milled cotton and Durafil were technically suitable for use, and have been found advantageous in improving growth of malignant cells and of enabling growth of normal and malignant cells to take place in fluid media.

We wish to thank Professor Astbury of Leeds for his advice, and Dr. T. K. Walker of the College of Technology, Manchester, for providing a culture of *Acetobacter aceti*. and advice on technique. We are indebted to Tootal Broadhurst Lee (Manchester), and in particular to Mr. J. T. Marsh, Mr. H. O. Williams and Miss A. C. Alexander, for nine samples of cellulose, including Viscose film. The Directors of the British Cotton Industry Research Association kindly supplied seven samples for testing, including colloid milled cotton and Durafil, also the electron micrograph (Fig. 6). For electron microscope studies of Viscose and

fibrin (Fig. 1) we thank Dr. A. S. Gomm and Mr. A. C. Cooper, Dyestuffs Division, I.C.I., Manchester.

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EXPLANATION OF PLATES.

FIG. 1.—Electron microscope study of fibrin in a plasma clot. × 7,000.

FIG. 5.—C3H mouse mammary tumour grown in a plasma clot with cellulose, colloid milled. Stained Giemsa. × 45.

FIG. 6.—Durafil broken up in a Waring Blendor gold-shadowed electron micrograph. × 12,000.

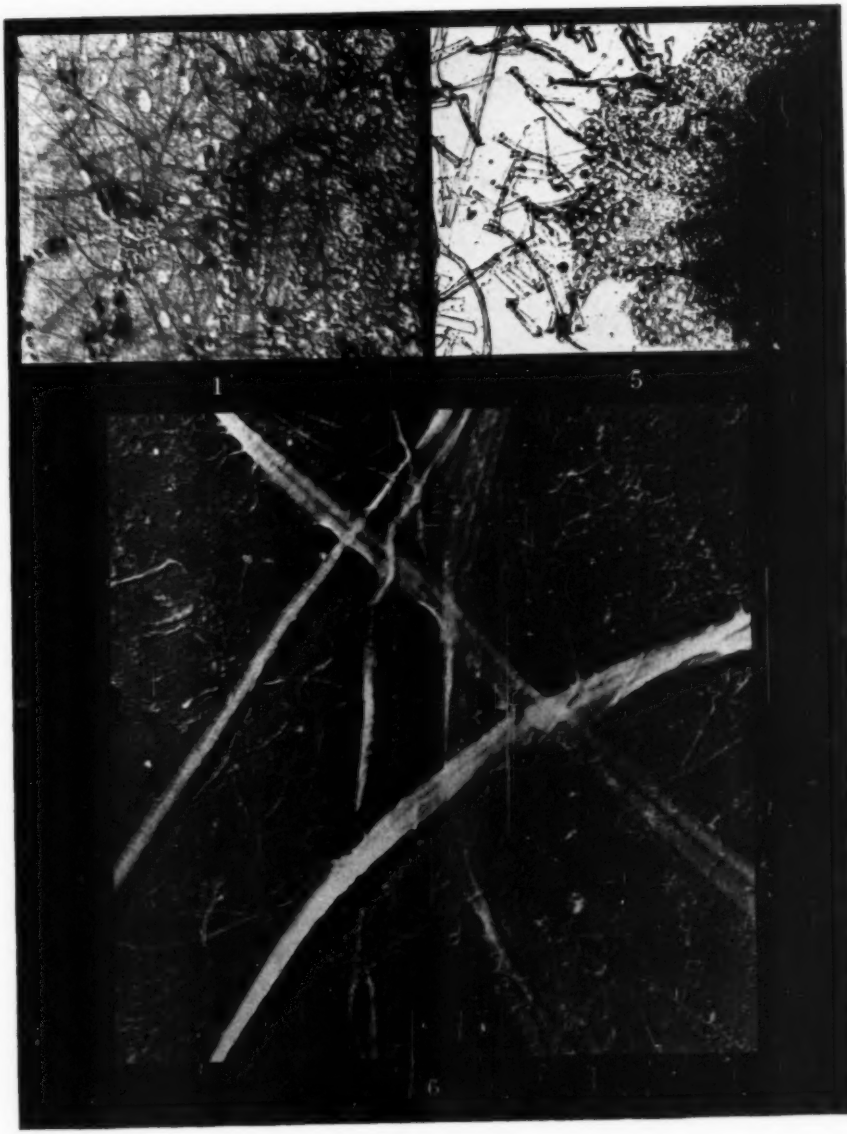
FIG. 7.—C3H mouse mammary tumour grown in plasma clot without fibres. Note uneven growth. Stained Giemsa.

FIG. 8.—Strong A mouse mammary tumour grown in a medium of equal parts mouse serum and hen plasma with Durafil fibres. Finer fibrils are present but do not show at this magnification. Stained Giemsa. × 22.

FIG. 9.—ABC mouse mammary tumour grown in liquid medium under Viscose film. Living culture unstained with film *in situ*. × 22.

FIG. 10.—48-hour culture of fibroblasts grown in liquid medium under Viscose film. Stained Giemsa. × 22.

FIG. 11.—ABC mouse mammary tumour grown in liquid medium under Viscose film. Stained Giemsa. × 22.



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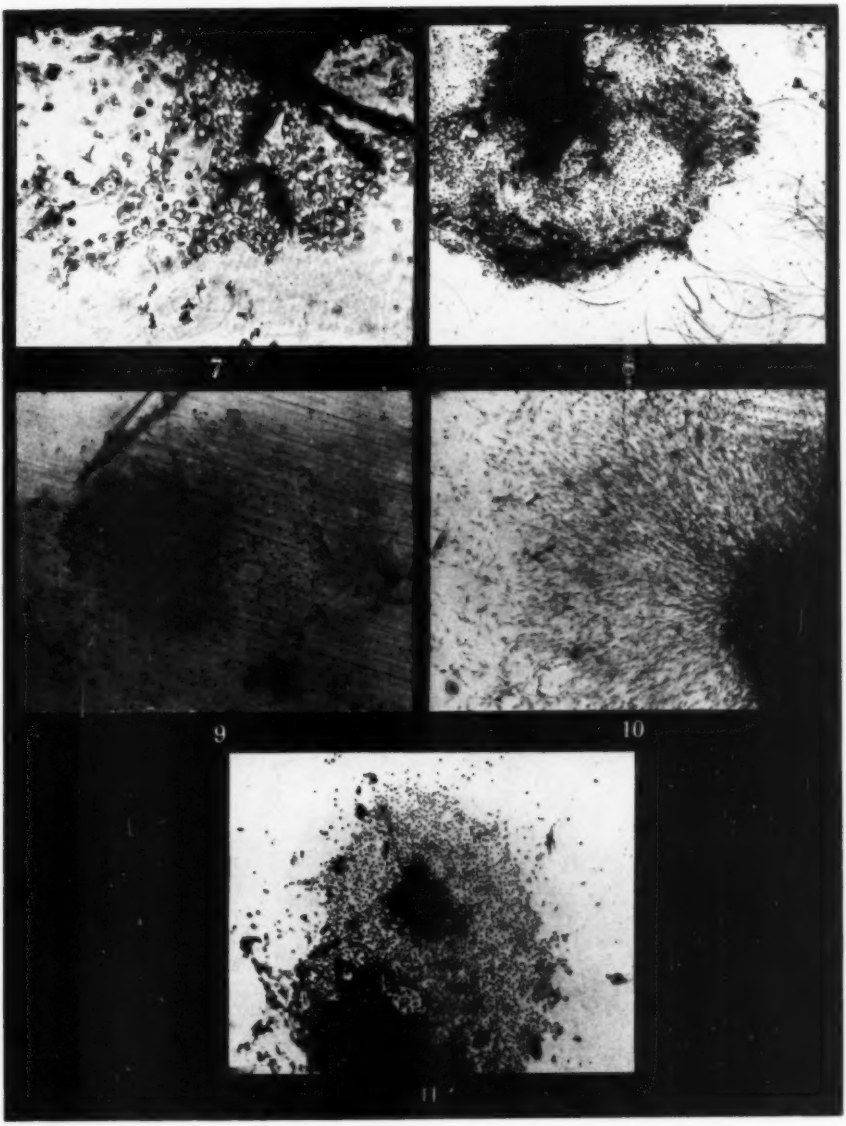

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